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Studies on Chromoproteins in Japanese Nori, *Porphyra tenera*

V. On the Sugar Components of Phycoerythrin

By TERUKO FUJIWARA

(*From the Institute for Protein Research, Osaka University, Osaka*)

(Received for Publication, September 30, 1960)

As described in the previous paper (*1*) phycoerythrin, a chromoprotein obtained from Japanese 'NORI' (*Porphyra tenera*) gives characteristic Molish and orcinol reactions, suggesting that the protein may be a glycoprotein*. Recently, the author found that several chromopeptides derived from phycoerythrin with proteinase, also possessed carbohydrate residues in such a high content that the presence could not be attributed to the contaminants (*2*, *3*). These facts indicate that phycoerythrin is a kind of glyco-chromoprotein and that the both prosthetic groups, carbohydrate and chromophore may exist closely to each other in the conjugated protein.

The present paper reports on the carbohydrates obtained from phycoerythrin, and from the chromopeptide P-12a (*2*) derived from phycoerythrin.

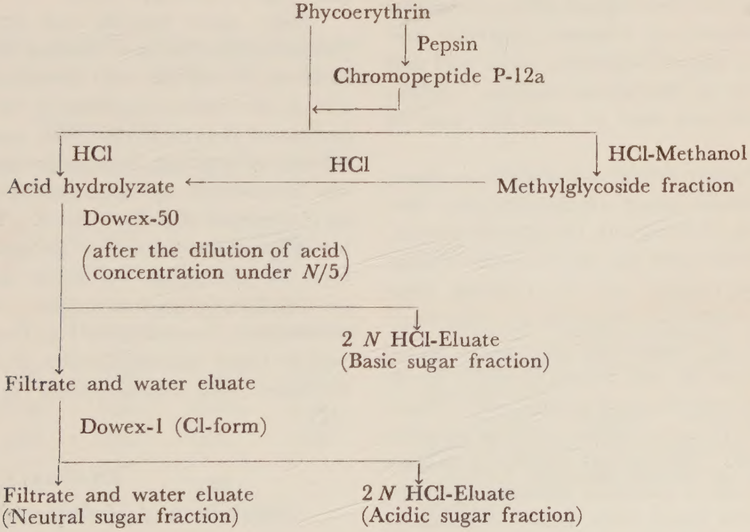
EXPERIMENTALS

Crystalline Phycoerythrin—Phycoerythrin was prepared from dried Japanese 'NORI' (*Porphyra tenera*) and was recrystallized three to four times, according to the method previously reported by the author (*1*).

Chromopeptide P-12a—Peptic hydrolysis of phycoerythrin, and the isolation of chromopeptide P-12a were achieved according to the method already descrived (*2*).

SCHEME I

Preparation of Carbohydrate Fractions from Phycoerythrin



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* Total sugar content of 4.8 to 5.4 per cent was obtained by colorimetry with orcinol (Unpublished).

Preparation of Carbohydrate Fractions—The general procedures used are summarized in Scheme I.

Acid hydrolysis of phycoerythrin was performed with $N/10$ to $2N$ hydrochloric acid at 100°C for a given period (2 to 20 hours). The filtrate was diluted with water and then was removed basic component (amino sugar, amino acid and peptide) according to the method shown in Scheme I.

Methanolysis was achieved in 3 per cent methanolic hydrogen chloride for a given period (29 to 78 hours), at the refluxing temperature. The resulting solution was directly neutralized with solid silver carbonate, and the filtrate from the insoluble materials, was concentrated to syrup in vacuum. A part of the residue was used as 3,6-anhydro-galactose dimethyl acetal faction for paper chromatographic analysis (*14*), while the another portion was further hydrolyzed with $2N$ hydrochloric acid at 100°C for 4 hours, and the resulting free sugars were fractionated according to the method shown in Scheme I.

Analytical Methods—Total reducing sugar content in acid hydrolyzate was determined according to the method of Somogyi (*4*, *5*) and was expressed as the glucose value.

The total sugar- (being expressed as the glucose equivalent), pentose-, glucuronic acid- and glucosamine-values were assayed by direct acid hydrolysis, and spectrophotometry with the use of orcinol (*6*)-, Bial (*9*)-, carbazole (*10*)- and Elson-Morgan (*7*)-methods, respectively.

Neutral sugars, xylose, arabinose and galactose, were identified by two dimensional paper chromatography, using mixtures of *n*-butanol, pyridine and water (6 : 4 : 3 v/v) and of *n*-butanol, acetic acid and water (4 : 1 : 1 v/v) as the solvent system. Aniline hydrogen phthalate was used to stain the spots of these sugars.

For the quantitative determination of these sugars, using one dimensional paper chromatography their spots corresponding to 10 mg. of the original protein, were separately eluted with one ml. of water without staining with color reagent, and the reducing sugar content of each extract was assayed as the followings:

A mixture of the eluate (one ml.) and 2 ml. of Somogyi's reagent (*4*) was heated at 100°C for 30 minutes, and was cooled to room temperature. Thereafter, 2 ml. of Nelson's reagent (*5*) was added to develop blue color. The optical density at 500 mμ was determined with a Beckman Spectrophotometer, after the dilution to 10 ml. with water. Preliminary experiments indicated that there existed a linearity between the optical density and the amount of the sugar applied on paper, in the range of 0 to 50 μg., and that the optical density produced from unit amount of each sugar was just the same, as shown in Fig. 1.

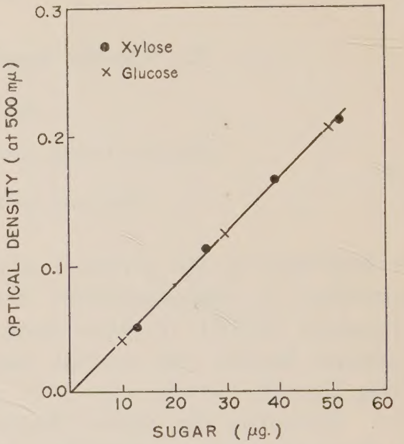

FIG. 1. Standard curves of reducing sugars estimated by paper chromatography and photometry with Somogyi's method.

Acidic sugar was analysed both by paper chromatography with the use of the above-mentioned solvent systems, and by paper electrophoresis which was performed for 4 to 5 hours under a potential of 600 volt with the use of $M/10$ phosphate buffer of pH 6.5, as the background electrolyte. For the detection of spot, naphthoresorcinol (*19*)- and *p*-anisidine (*20*)-reagents were also used.

Basic sugar fraction was analysed by elution chromatography from a column of Dowex-50X8 (H-from) of 0.6×43 cm. with the use of $N/5$ hydrochloric acid as the eluate, according to the slightly modified method of Gardell (*8*). The sugar content in the effluent which had been collected in 2 ml.-fraction, was determined by spectrophotometry with Somogyi's method described above. The standard curve for glucosamine was also in good agreement with those for glucose and for xylose (see Fig. 1).

3,6-Anhydro-galactose was analysed both by polarography according to Fujiwara's method (*16*) and by paper chromatography of the dimethyl acetal derivative with *o*-aminophenol as the color reagent (*14*).

RESULTS

Sugars in Acid Hydrolyzate of Phycoerythrin—On the acid hydrolysis of phycoerythrin, appreciable amounts of sugar were liberated. These contents were determined on the hydrolyzates under various conditions after removing of basic component by Dowex-50 column, with Somogyi's, orcinol-, Bial's and carbazole-methods. These results are summarized in Table I. It should be noted that the orcinol- and carbazole-values were rather close (3.5 to 4.8 per cent; 3.1 to 4.9 per cent), while the reducing sugar contents were fairly lower than the former two (0.5 to 1.4 per cent).

TABLE I

Sugar Contents of Acid Hydrolyzate of Phycoerythrin

Conditions of hydrolysis	Orcinol-H_2SO_4 1)	Somogyi 2)	Bial 3)	Carbazole 4)
	%	%	%	%
0.1 *N* HCl 3 hrs.	4.78	1.06	1.50	4.89
0.1 *N* HCl 20 hrs.	3.86	1.28	0.46	3.06
1 *N* HCl 20 hrs.	3.54	0.52	—	—
2 *N* HCl 2 hrs.	3.96	1.35	1.06	3.33

1) Glucose value
2) Reducing sugar value
3) Pentose value
4) Glucuronic acid value

The reducing sugars, after the removal of basic components by Dowex-50 column were analysed by paper chromatography. As shown in Fig. 2, there were observed two main components which were identified as

TABLE II

Reducing Sugar Contents in Acid Hydrolyzate of Phycoerythrin

	0.1 *N* HCl 3 hrs.		2 *N* HCl 4 hrs.	
	%	Moles/10^5g. of protein	%	Moles/10^5g. of protein
Total sugar 1)	1.00	5.53	1.42	7.89
Galactose	0.34	1.89	0.42	2.31
Xylose	0.32	2.12	0.45	2.98
Hexosamin 2) fraction	0.16		0.08	

1) Glucose values by Somogyi's method
2) Glucosamine values by Elson-Morgan's method

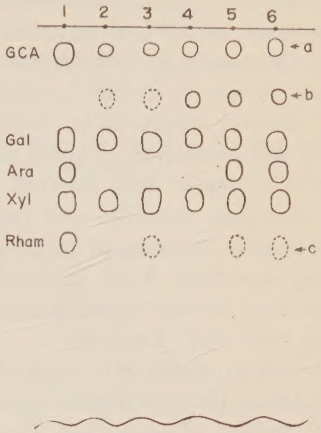

FIG. 2. One dimensional paper chromatograms of reducing sugars obtained from phycoerythrin*

1: Authentic sugars

2–3: Sugars in the hydrolyzate of phycoerythrin (2) with *N*/10 HCl for 3 hours, (3) with 2 *N* HCl for 4 hours

4–6: Sugars in the hydrolyzate of (4) 29 hours-, (5) 53 hours-, (6) 60 hours-methanolyzate of phycoerythrin.

a, b and c: see the test.

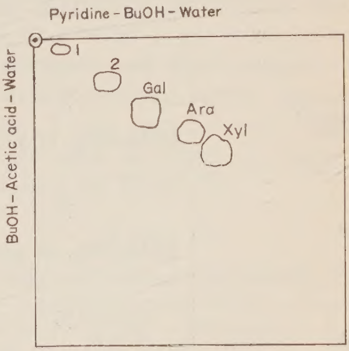

FIG. 3. Two-dimensional chromatogram of sugars obtained from phycoerythrin after 60 hours methanolysis.*

Solvent systems *n*-butanol-pyridine-water (6:4:3 v/v) and *n*-butanol-acetic acid-water (4:1:1 v/v). The spray reagent was aniline-hydrogen-phthalate.

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* The following abbreviation have been employed: GCA=Glucuronic acid, Gal=galactose, Ara=arabinose, Xyl=xylose, Rham=rhamnose.

galactose and xylose, together with two or three minor components according to their R_f values. Table II summarizes the amounts of total reducing sugar, galactose and xylose in rather short period-hydrolyzates.

Sugar Components in Methanolyzate of Phycoerythrin—As shown in Table I, reducing sugar contents showed appreciable increase on a prolonged acid hydrolysis. In this experiment, phycoerythrin was at first treated with methanolic hydrogen chloride, and the resulting methanol-soluble fraction of methylglycosides after being separated from insoluble protein fragments was further hydrolyzed with hydrochloric acid (2 *N*, 4 hours).

As shown in Table III, methylglycosides liberated from phycoerythrin were also not so stable during a prolonged methanolysis. However, the amounts of reducing sugar obtained by methanolysis (maximum 1.9 per cent) was somewhat higher than that in direct acid hydrolyzate (maximum 1.4 per cent). Most notable fact was the presence of arabinose as an additional main component, together with xylose, galactose and two or three minor components, which had not been detected in direct acid hydrolyzate. These results are shown in Figs. 2, 3 and Table III.

The minor component C which was detected both in direct acid hydrolyzate and in hydrolyzate after methanolysis, could not be distinguished from rhamnose by their chromatographic behaviors, and showed a color with Dische's reagent (*11*). However, the absorption spectrum was quite different from that of rhamnose. The both absorption spectra are shown in Fig. 4. The contents of this component in direct acid hydrolyzate and in hydrolyzate after methanolysis, was estimated only 0.45 and 0.58 moles per 10^5 g. of phycoerythrin, respectively.

The minor component B shown in Fig. 2, seemed to be a disaccharide composed of galactose as it possessed a fairly low R_f values, and the extract from paper gave galactose and one more sugar by rehydrolysis. No another sugar is identified. The reducing power indicated 2 times of originals after the

TABLE III

Reducing Sugar Content in Methanolyzate of Phycoerythrin

	Time (hours)	29	53	59	60	64	78
Total Sugar[1]	%	0.816	1.042	1.310	1.900	1.265	0.438
	Moles/10^{5}g. of protein	4.52	5.76	7.25	10.55	7.04	2.43
Galactose	%	0.244	0.271	0.318	0.312	0.400	0.128
	Moles/10^{5}g. of protein	1.35	1.51	1.77	1.75	2.22	0.71
Arabinose	%	none	0.218	0.230	0.236	0.237	0.162
	Moles/10^{5}g. of protein		1.45	1.53	1.57	1.58	1.08
Xylose	%	0.204	0.252	0.342	0.456	0.237	0.051
	Moles/10^{5}g. of protein	1.36	1.68	2.28	3.05	1.58	0.34
Hexosamine fraction[2]	%		0.089		0.116	0.137	

1) Glucose values by Somogyi's method
2) Glucosamine values by Elson-Morgan's method

re-hydrolysis.

The minor component A was fractionated as acidic compound with a column of Dowex-1, and colored with both naphthoresorcinol- and *p*-anisidine-reagents. By paper electrophoresis, the compound also migrated as a single spot to anode, but with an electromobility different from those of usual uronic acids. The amount was as low as 0.12 to 0.45 moles per 10^5 g. contrary to a very high uronic acid-values estimated by carbazole method (Table I). It is doubtful whether this acidic components is a real uronic acid or not.

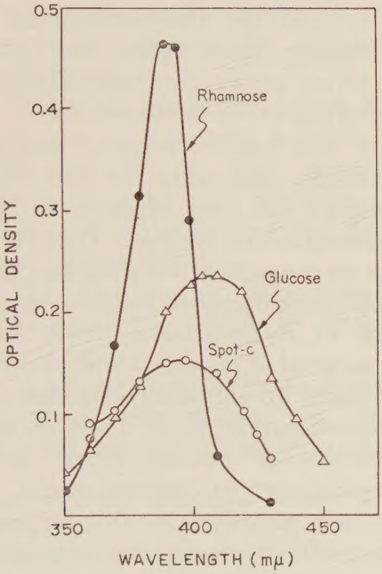

Fig. 4. Absorption spectra of the products of the Dische reaction on spot-C obtained by 60 hrs. methanolysis.

Some amounts of amino sugar were observed by hydrolysis, and photometry with Elson-Morgen method, thus 0.08 to 0.16 per cent in the direct acid hydrolyzate, and 0.14 per cent in the hydrolyzate after methanolysis (Tables II and III). Elution chromatography of the basic fraction, however, indicated no sign of the presence of glucosamine, nor of galactosamine. The result is shown in Fig. 5.

3,6-Anhydro-galactose dimethyl acetal fraction derived from phycoerythrin with methanolysis, was examined by paper chromatography, and there was observed a faint yellow spot colored with *o*-aminophenol reagent (*14*). The content in this fraction, however, was too low to be determined by polarography (*16*), if it were realy 3,6-anhydro-galactose.

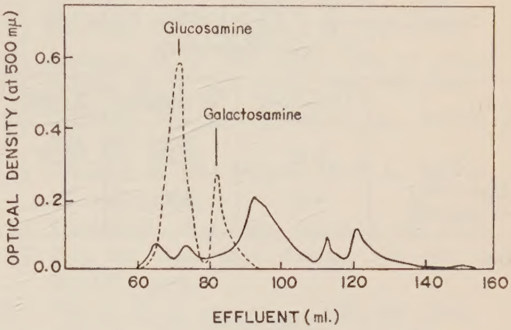

Fig. 5. Elution pattern of hexosamine fraction of phycoerythrin from Dowex-50X8 (H^+ form), 0.6×43 cm. column eluted with 0.2 *N* HCl solution.
—— Acid hydrolysis (2 *N* HCl, HCl, 4 hours.)
--- Authentic hexosamine, glucosamine and galactosamine.

Sugar Components of Chromopeptide P-12a—Chromopeptide P-12a* obtained from phycoerythrin by peptic hydrolysis, had been presumed to possess sugar component from the low nitrogen and high oxygen contents (*2*). On acid hydrolysis, there appeared approximately 4 per cent of total sugar, but only 1.4 per cent of reducing sugar. The result is shown in Table IV.

Table IV

Total Sugar and Sulfur of Chromopeptide, P-12a

Conditions of hydrolysis	Orcinol-H_2SO_4 (%)	Somogyi (%)	Sulfur[1] (%)
0.1 *N* HCl 3 hrs.	1.33 1.38	— —	0.84 —
2 *N* HCl 2 hrs.	3.89	1.38	0.85

1) Sulfur in sugar fraction

The reducing sugar components were examined by methanolysis, and further hydrolysis of the methylglycoside fraction, according to the procedure already applied to phycoerythrin. There were observed three sugars, xylose, arabinose and galactose in the neutral sugar fraction, too. The contents are summarized in Table V.

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* Minimum molecular weight; 2,145 (*2*)

TABLE V

Sugar Components of Chromopeptide, P-12a after 70 Hours Methanolysis

	Galactose	Arabinose	Xylose
%	0.87	0.56	0.31
Moles/10^5*g.* of peptide	4.9	3.7	2.1

DISCUSSION

The total sugar- and uronic acid-contents of phycoerythrin were estimated to be 4.78 and 4.89 per cent by acid hydrolysis, and photometry with orcinol and carbazole, respectively (Table I) while a value of 4.76 to 6.40 per cent in total sugar had been obtained by direct photometry of intact phycoerythrin with orcinol.* These results indicate that the protein possesses sugar components at a concentration of 4.8 to 5.4 per cent, thus at a ratio of 28 to 31 moles per 10^5g., and that the most part of sugar components may be uronic acid-like substance.

The total reducing sugar content, on the other hand, was only 1.42 per cent on the direct acid hydrolyzate, and 1.9 per cent on the hydrolyzate of methylglycoside fraction derived from phycoerythrin with methanolysis. Thus these amounts were as low as one third to one fourth of the total sugar content. Three reducing sugars, xylose, arabinose and galactose, were identified (Figs. 2 and 3) by paper chromatography. The amounts of 0.46 per cent in xylose (3.1 moles per 10^5g.), of 0.24 per cent in arabinose (1.6 mole per 10^5g.) and of 0.40 per cent in galactose (2.2 moles per 10^5g.) were not so inconsistent with the total-reducing sugar content mentioned above and with the total pentose content estimated with Bial's method (1.1 to 1.5 per cent).

Besides of these three sugars, one acidic and one neutral sugars were also detected as minor components by paper chromatography. The low content of free acidic sugar, but the high uronic acid value in the acid hydrolyzate of phycoerythrin, may suggest that the acidic sugar residues firmly bound with the protein moiety. No arabinose could be detected in the direct acid hydrolyzate and in the hydrolyzate after short period-methanolysis (Tables II and III). The fact seem to indicate that the sugar is also firmly combined with the protein moiety directly or via other residue such as the above-mentioned acidic sugar, differing from xylose and galactose residues which were rather easily liberated by acid hydrolysis or by methanolysis.

As is well known, seaweed was rich in polysaccharide, and some of red seaweed polysaccharide has been reported to contain 3,6-anhydro-galactose residue. Thus Hibino (*18*) reported the presence of galactose, glucose, ketose and methylpentose residues in a polysaccharide of *Porphyra tenera*, which was the starting material of phycoerythrin, and Nunn (*12*) has found 3,6-anhydro-L-galactose residue in a polysaccharide from *Porphyra capensis*. The presence of glucose, ketose and 3,6-anhydro-galactose in phycoerythrin, may exclude the posibility in this experiment. According to Hibino (*18*) the polysaccharide of this seaweed, *Prophyra tenera*, is mainly composed of DL-galactose. So, in this connection, it will be of interest that phycocyanin—another chromoprotein in *Porphyra tenera*—contains DL-galactose which was confirmed by the stereospecific fermentation of yeast, *Saccharomyces sake*.*

From these results, it is speculated that the galactose of phycoerythrin may be also DL-form.

Chromopeptide P-12a derived from phycoerythrin with pepsin (*2*) also possessed xylose, arabinose and galactose. Their contents shown in Table V, were obtained on the hydrolyzate of methylglycoside fraction derived with a prolonged methanolysis. So their lower values cannot exclude the presumption that the chromopeptide will be a glyco-chromopeptide, and the both prosthetic groups, chromophore and carbohydrate parts exists fairly close to each other.

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* Unpublished (T. Fujiwara).

* Unpublished (T. Fujiwara).

SUMMARY

1. Total sugar content of crystalline phycoerythrin was estimated to be 4.78 per cent (28 moles per 10^5g.) by acid hydrolysis, and photometry with orcinol.

2. More than two thirds of the sugar components seemed to be uronic acid-like substance which was liberated from phycoerythrin only with difficulty by acid hydrolysis or by methanolysis.

3. Three neutral sugars, xylose, arabinose and galactose were isolated in yields of 0.46, 0.24 and 0.40 per cent from phycoerythrin, respectively, together with much less amount of uronic acid-like substance and of an unidentified neutral sugar.

4. No glucosamine, galactosamine and 3,6-anhydrogalactose could be detected in phycoerythrin.

5. A chromopeptide, P-12a derived from phycoerythrin with pepsin, also contained xylose, arabinose and galactose residues.

This work was performed in the Institute of Chemistry, Faculty of Industrial Arts, Kyoto Technical University, under the direction of Prof. C. Araki and in the Institute for Protein Research, Osaka University, under the direction of Prof. S. Akabori.

The author wishes to express her sincere gratitude to Prof. C. Araki and Prof. S. Akabori for their constant interest and encouragement.

She also wishes to thank Mr. K. Arai for his valuable suggestions and technical help and Miss K. Okada for technical assistance.

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Amino Acids in the Brain

Alteration of Amino Acids in Rabbit Brain Caused by Repetitive Convulsive Fits and Comparison of Amino Acid Contents in Epileptic and Non-epileptic Human Brain

By YASUHISA YAMAMOTO, AKITANE MORI and DENNOSUKE JINNAI

(From the Department of Surgery and Neurosurgery, Okayama University Medical School, Okayama)

(Received for publication, October 11, 1960)

With a remarkable advance in ion exchange resin column chromatography, its application has become quite advantageous for the analysis of amino acids. Moore and Stein successively reported in 1952 the methods of analysis of total amino acids by aid of 2 columns of Dowex-50 X8 (one for the analyses of the acidic and neutral and the other for basic amino acids) (*1*), in 1954 the method with 150 cm. column of Dowex -50 X4 (*2*), and more recently (1958) the "Improved System" (*3*) (*4*) using 2 columns of Amberlite IR-120. The materials analysed are always added to Na form column of strong acid ion exchange resin and analysed with buffer of adequate pH and ionic strength. Among them, the method which was reported in 1952 is suitable for analysing fluid mixture consisting of only amino acids such as completely hydrolysed products of protein, whereas the methods reported in 1954 and 1958 are suitable for chromatography not only of amino acids but of its related substances and various peptides in body fluid and in every tissue. For the elucidation of the influence of convulsion caused by electrical stimulation upon free amino acids in rabbit brain and for the comparison of amino acid contents in the epileptic and non-epileptic human brains, the amino acids in brains were analysed by the Improved System using the 150 cm. and 50 cm. columns in the present experiment.

EXPERIMENTALS

Resin—Amberlite IR-120 (400–600 meshes) is treated with 4 *N* hydrochloric acid and 2 *N* sodium hydroxide as stated in (*3*). The resin is divided into 5 fractions according to hydraulic separation by the method of Hamilton (*5*), and in Table I is given the fractionation of lots of Amberlite IR-120. The resin used for 150 cm. column is C fraction and for 50 cm. column B fraction.

TABLE I

Description of Fractions of Amberlite IR-120

Fraction	Water Flow (2-Liter Funnel) ml./min.	Use of Resin
A	50	Not used
B	50–110	15 and 50 cm. Columns
C	110–280	150 cm. Column
D	280–580	Not used
E	Residue	Not used

Preparation of Ion Exchange Columns—The ion exchange columns are prepared according to the description in (*3*) and the pressure at the pouring of resin is 20 cm. Hg.

Buffers—The buffers as mentioned in Table II are used. They are similar to the buffer stated in (*3*) but without addition of thiodiglycol.

Pressure and Flow Rate—The pressure of 18–20 cm. Hg is applied on 500 ml. separatory funnel. The flow rate of the long column is 8–18 ml./hour and that of the short column is 10–12 ml./hour. A pressing equipment, which utilized the difference in water head as stated in Fig. 1, is used. The pressure is kept at the atmospheric pressure plus the difference in water head.

Materials—(A) White rabbits about 2 kg. in body weight are used. Through one week electrical stimulation is given 4 to 5 times a day. The stimulation is given in the following manner: 1/3 needle electrodes are laid on both sides of the temporal region and charged with 20 V for 2 sec., thus convulsion is provoked. The sagital decapitation is done 24 hours after the last electrical stimulation. The brain is taken out immediately and frozen in dry ice acetone. The pia mater and blood vessels are removed and only the cerebral cortex is used. (B) The human brain is excised during surgical operation under necessity of clinical indication. As a contrast, that of non-epileptics, *i. e.* brain tumor and others, are selected. These are frozen in dry ice acetone as soon as they are taken out and only the cortex is studied. As for the epileptics, brain of epileptics, which is excised from those parts where we find focus in electro-corticogram is used.

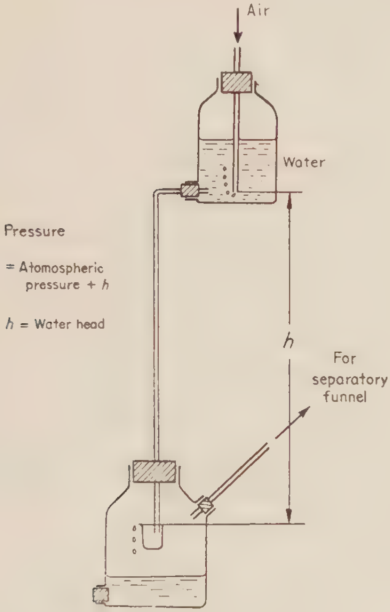

Fig. 1. Pressing equipment.

Preparation of Tissue Extracts—Protein free extracts of the tissues are preparaed with aqueous picric acid and the picric acid is removed as described elsewhere (*6*).

Manipulation of the Columns—One gram of tissue extract (protein free extract of 1 g. brain) is put into the buffer (0.2 *N*), pH 2.2, making the volume 5 ml. One ml. of it is added to the top of the column. With 150 cm. column, the initial buffer (0.2 *N*) is at pH 3.25 and the column is maintained at the temperature of 30°C and effluent is collected in 2 ml. fractions. After the elution of the peaks of glutamic acid and glycine (*ca.* 210 *ml.*) with buffer (0.2 *N*), pH 4.25, kept at 50°C, neutral amino acid is eluted. In this way the effluent curve as illustrated in Fig. 2-A is obtained. In utilizing 50 cm. column the same material is treated with buffer (0.38 *N*), pH 4.26, at 30°C and chromatographed. Following the peak of acidic and neutral amino acid, the peak of GABA (γ-aminobutyric acid) is effluented at about 160 ml. Then amino acids are eliminated as illustrated in Fig. 2-B but the amount proves to be neglible.

RESULTS AND DISCUSSION

Recovery Rate and Identification—For recovery rate, as was the case for the Dowex -50 X4 method (*2*), analysis of a known mixture yields the value 100 plus or minus 3 per cent, except methionine. This result is almost the same as that in reference (*4*). Methionine is

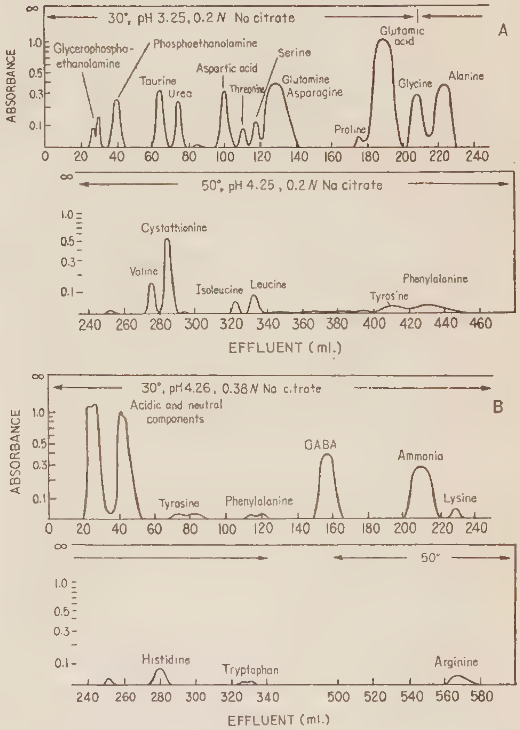

Fig. 2. Chromatographic analysis of extract of non-epileptic brain.

In upper figure; glutamine emarged in the same rate 120–140 ml. with asparagine. Serine emarged sometimes overlapping in the same zone. In order to elute argine more rapidly, a simultaneous change to a 0.38 *N* buffer at pH 6.5 (Table II) will bring arginine off the column even erlier (at about 500 ml.).

TABLE II

Preparation of Sodium Citrate Buffers

pH	Sodium Concn., *N*	Citric Acid H_2O, g.	NaOH (97%), g.	HCl (Concd.), ml.	Final Vol., liters	Phenol Added of Final Vol., g.
2.2±0.03	0·20	105	42	80	5	0
3.25±0.01	0.20	840	330	462	40	40
4.25±0.02	0.20	840	330	188	40	40
4.26±0.02	0.38	532	312	307	20	20
6.50±0.05	0.38	133	78	6.3	5	5

Before use, add 5 ml. of Emargen S[1] per liter

[1] The solution is prepared by dissolving 50 g. of Emargen S 120 (admixture for surface tension) in 100 ml. of hot water.

not quantitatively analysed because it is decomposed. Identification of the substances responsible for various peaks is carried out according to the descriptions in reference (*7*).

Calculation—The effluent is collected in 2 ml. fractions employing fraction collector. The coloring is done according to a modified ninhydrin method (*8*), while quantitative analysis is from the standard curve using L-leucine—a standard curve is plotted in the manner described in (*9*).

Data—Data are summarized in Tables III and IV. Glutamine has the same rate of travel as asparagine. But as glutamine is oxygenated at room temperature (*10*) and has a low recovery rate and the amount of asparagine is very scanty, their amount is not estimated. Threonine and serine often overlapped one another. In the case where such overlapping occurs, no estimation is attempted. Tyrosine and phenylalanine are omitted from data because they are very small in quantity and eluted simulataneously. Basic amino acids eluted in 50 cm. column are a illustrated in Fig. 2-B.

Among them only GABA and NH_3 are shown as big peaks but other amino acids are not estimated because our main interest lies in GABA, and the peak of GABA is demonstrated quite markedly when compared with other amino acids. From these results it can be recognized that the amino acid contents differ depending upon the part where material is taken as shown in Table IV. The similar data are obtained in all the five cases because the extracts from homogenized total cortex are used in the case of rabbit brain. Therefore, only mean values are summarized in Table III. On the other hand, the specimens have to be taken out from different areas in each patients in the series of human brains, because they are

TABLE III

Amino acid	Non affected	Affected
Glycerophosphoethanolamine	1.384	1.425
Phosphoethanolamine	1.071	1.156
Taurine	1.288	1.970
Urea	5.024	6.980
Aspartic acid	2.639	2.390
Threonine	0.309	0.410
Serine	0.969	1.000
Glutamic acid	8.375	12.578
Glycine	0.856	1.906
Alanine	1.701	1.320
Valine	0.462	0.450
Cystathionine	0.170	0.152
Isoleucine	0.160	0.155
Leucine	0.190	0.190
GABA	2.318	1.195

(Average of 5 cases)

The figure indicates unit: μmole/g. Affected: electrically stimulated rabbits.

TABLE IV

Free Amino Acids in Human Brain

case	Non epileptic						Epileptic					
	W. ♀	O. ♀	S. ♂	M. ♀	E. ♀		S. ♂	I. ♂	N. ♂	Y. ♀	T. ♂	
part / Amino acid	frontal	frontal	temporal	occipito-temporal	fronto-parietal	A. V.	temporal	temporal	temporal	temporal	fronto-temporal	A. V.
Glycerophosphoethanolamine	1.386	0.508	1.116	2.337	1.155	1.300	3.218	1.493	1.614	1.386	1.512	1.845
Phosphoethanolamine	3.685	1.192	1.477	2.874	1.481	2.142	2.165	0.595	0.986	2.439	1.117	1.465
Taurine	1.236	1.427	1.672	0.975	2.805	1.623	1.765	1.410	1.493	1.428	1.510	1.521
Urea	18.528	11.500	13.410	10.287	19.850	14.219	16.077	10.110	10.997	11.500	9.776	11.692
Aspartic acid	2.306	2.018	3.017	2.130	2.230	2.340	3.423	2.298	2.808	3.018	3.123	2.934
Threonine	0.803	0.754		1.048	0.700	0.826	0.745	0.696	0.706	0.711	0.752	0.722
Serine	0.911	0		1.217	0.958	1.029	0.818		0.794	0.800	0.874	0.822
Glutamic acid	8.834	9.790	9.403	8.905	8.096	9.006	11.915	9.793	11.748	9.794	10.649	10.780
Glycine	0.707	0.901	1.837	0.855	1.050	1.070	0.476	0.536	0.512	0.701	0.642	0.573
Alanine	0.651	0.609	0.710	0.676	0.665	0.622	0.572	0.649	0.639	0.779	0.701	0.668
Valine	2.688	1.435	0.455	0.715	0.466	1.152	0.084	0.140	0.160	0.188	0.135	0.141
Cystathionine	2.368	3.488	0.828	1.999	2.041	2.145	1.375	1.451	2.957	1.510	1.900	1.838
Isoleucine	0.560	0.338	0.583	0.473	0.606	0.512	0.410	0.385	0.396	0.338	0.357	0.377
Leucine	0.476	0.312	0.443	0.391	0.408	0.406	0.342	0.315	0.326	0.340	0.310	0.327
GABA	7.636	7.620	7.898	8.799	6.965	7.784	6.073	5.126	6.750	6.810	6.105	6.173

unit: μmole/g.

In epileptic case of I ♂ and nonepileptic cases W ♀, S ♂ and ♀ intravenous drop infusion of urea was performed during operation, for the purpose of preventing brain edema. The blanc parts are the parts of overlap.

excised under different conditions necessarily in various surgical operations. Moreover, when the differences in the general conditions in different individuals are taken into account, it is obvious that the contents of amino acids would have naturally shown various values. But only in the case of epileptic brain, the materials are secured from motor areas and epileptic focus, so it seems that similar values easily can be obtained. The epileptic brain contains more glutamic acid than that in non-epileptic ones. The difference between motor area and non-motor area needs to be taken into consideration, but because there can be found an increase in glutamic acid also in rabbits with convulsion, it is reasonable to assume that the result is probably true. The amount of glycine in the case of rabbits is larger in those with convulsion. But the epileptics show one half of glycine in the non-epileptics. Alanine is slightly decreased in rabbits with convulsion and also it is reduced to one half in the epileptics. Cystathionine exists in rabbit brains in a negligible quantity. Tallan, Moore, and Stein (*11*) scrutinized this fact in various animal tissues and reported that cystathionine is contained relatively in a greater quantity in the human brain. This substance, as illustrated in Table IV, shows various values and it can not be decided whether this result depends on the difference in areas or on a smaller content in the motor area. It is assumed that this substance is contained less in the motor area, and in addition, this phenomenon may be influenced by the epileptic state. It is well known that this substance is an intermediate substance between methionine and cysteine (*12*). Decomposition of cystathionine is restrained under deficiency of vitamin B_6, and in such a state it is accumulated in the body and later excreted because cystathionase is a pyridoxal dependent enzyme (*13*, *14*). This problem demands more precise investigation and instigates a keen interest in relation to vitamin B_6 deficiency. GABA shows a decrease in rabbits with convulsion and epileptics. It is considered that an increase of glutamic acid and a decrease of GABA in epileptic brain are caused by the decreased activity of glutmaic decarboxylase. It is supposed that idiopathic epilepsy must be caused by the disturbance in the metabolism of GABA or in the related substances. The results are worthy of notice from the fact that in the epileptic brain there exists enzymatic defect in the stage of glutamic decarboxylase which has a direct relation to GABA formation. It has been clarified that the epileptic brain has an abnormality in amino acid contents in different areas of the brain. This problem requires further studies for the clarification.

SUMMARY

In order to clarify the cause of epilepsy, analyses of free amino acids in the brain were performed by various methods, and the following results were obtained.

1. Glutamic acid is contained in a greater quantity in the epileptic brain than in the non-epileptics.

2. The contents of glycine and alanine are less in the epileptic brain.

3. The cystathinonine contents seem to be less in the epileptic brain.

4. GABA is contained in a smaller quantity in the epileptic brain.

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The Relation of Alanine and Vitamin B_6 to Growth of Lactic Acid Bacteria*

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The utilization of several stereoisomers of natural amino acids by some lactic acid bacteria in the presence or absence of alanine and vitamin B_6, has been studied by the authors. During the course of these studies, it was observed that *Leuconostoc mesenteroides P-60* and *Lactobacillus arabinosus 17-5* and also *Streptococcus faecalis R*, which is known to require vitamin B_6 for growth, grew well in the absence of the vitamin B_6. This growth is assumed to be due to a large amount of D-alanine contained in the basal medium as previously noted by Snell (*1*).

Several reports have been published (*1-4*) concerning the relationship of alanine and vitamine B_6 to growth of lactic acid bacteria. But these studies were carried out under some limited or ambiguous conditions. Namely the medium deficient in vitamins (*4*) or the medium containing casein hydrolysate was employed instead of chemically defined mixture of amino acids (*1–3*). Therefore, the relationship of D- or L-alanine and vitamin B_6 to the growth of lactic acid bacteria was not clarified by these studies since the casein hydrolysate contains L-alanine.

In order to elucidate the above interrelationships, extensive investigations on the alanine and vitamin B_6 requirements of the lactic acid bacteria were carried out by employing chemically defined synthetic media.

EXPERIMENTS

Test Organisms and Stock Culture—Streptococcus faecalis R, Leuconostoc mesenteroides P-60 and *Lactobacillus arabinosus 17-5*, were used for the growth tests. These were maintained by weekly transfer as stab cultures in a medium composed of 1.5% agar, 0.5% yeast extract, 1% meat extract and 1% peptone. All cultures were incubated at 37°C until good growth occurred and stored in a refrigerator until the next transfer.

Culture Medium—The composition of the basal medium used in this experiment is shown in Table I. This composition was essentially the same as that of Henderson-Snell (*5*) and the L-forms of amino acids were mainly employed.

Inoculum—Inocula were prepared by transfering from the stock culture and grown for 17 hours at 37°C in the basal medium supplemented with DL-alanine 200 mg., pyridoxine-HCl 200 μg., pyridoxamine-2HCl 60 μg. and pyridoxal-HCl 60 μg. per 100 ml. of the double strength medium. The cells were centrifuged, washed three times and resuspended in 10 ml. of sterile 0.9% sodium chloride solution and one drop of suspension was used for each assay tube.

Assay Procedure—Assays were carried out in 15× 150 mm. test tubes covered with aluminium caps and in a total volume of 2 ml.. The tubes were autoclaved for 3 minutes at 1 kilogram pressure for sterilization. Vitamin B_6 compounds were autoclaved separately and then added to the sterilized basal medium befor use, because of the lability of pyridoxal when this vitamin is autoclaved with amino acids (*6*). One drop of inoculum was added to each assay tube from a sterile syringe. The tubes were then incubated at 37°C for 24 hours and the cell growth were turbidimetrically estimated by putting the culture tubes in the Hitachi EPO-B electric photometer at 660 mμ; the results are expressed as optical density.

RESULTS

Growth Response of Str. faecalis R to Alanine on Vitamin B_6-Free Medium—A comparison of the growth response of *Str. faecalis R* to L-alanine, D-alanine and DL-alanine in the absence of vitamin B_6 is shown in Fig. 1.

Str. faecalis R could not grow in the ab-

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* Presented at the 2nd Symposium of Microbioassay, Kyoto, February 6, 1960.

TABLE I

Composition of Basal Medium

Component	Amount per 100 ml. double strength medium	Component	Amount per 100 ml. double strength medium
Glucose	4,000 mg.	L-Proline	20 mg.
Na-Acetate·$3H_2O$	330	DL-Serine	40
Na-Citrate	4,000	L-Threonine	20
NH_4Cl	600	L-Tryptophan	20
K_2HPO_4	1,000	L-Tyrosine	20
$MgSO_4 \cdot 7H_2O$	160	L-Valine	20
$FeSO_4 \cdot 7H_2O$	8	Thiamine·HCl	200 μg.
$MnSO_4 \cdot 7H_2O$	32	Ca-Pantothenate	200
NaCl	8	Riboflavin	200
(DL-Alanine)	(200)[1)]	Niacin	200
L-Arginine·HCl	40	*p*-Aminobenzoic acid	40
L-Aspartic acid	100	Biotin	2
L-Cystine	20	Folic acid	2
L-Glutamic acid	200	Adenine·$H_2SO_4 \cdot 2H_2O$	2
Glycine	20	Guanine·HCl·$2H_2O$	2
L-Histidine·HCl	20	Uracil	2
L-Isoleucine	20	Xanthine	2
L-Leucine	20	(Pyridoxine·HCl)	(200)[1)]
L-Lysine·HCl	40	(Pyridoxamine·2HCl)	(60)[1)]
L-Methionine	20	(Pyridoxal·HCl)	(60)[1)]
DL-Phenylalanine	40		

1) DL-Alanine and vitamin B_6 were omitted from the complete basal medium. When vitamin B_6 or DL-alanine was supplemented, the parenthesized quantities were added unless otherwise noted. The medium was adjusted to pH 6.8 before use.

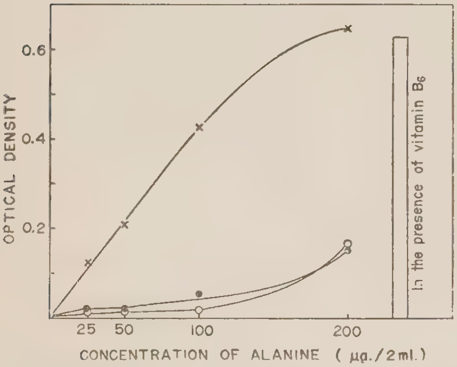

FIG. 1. Growth response of *Str. faecalis R* to alanine isomers in the absence of vitamin B_6.
Incubation was carried out for 24 hours at 37°C. —○— L-alanine, —●— D-alanine, —×— DL-alanine

sence of alanine but grew in parallel with the concentration of DL-alanine. Consequently it is apparent that this organism requires either alanine or vitamin B_6 for its growth. The growth in the medium containing more than 200 μg. of DL-alanine was the same as that in the medium supplemented with vitamin B_6. These results are the same as that obtained by Ogawa (*4*) with the medium limited in vitamins. Measurable growth was not observed with low concentrations of D-alanine or L-alanine, although slight growth occurred at higher concentrations. Effect of the addition of L- or D-alanine to the medium containing either D-alanine or L-alanine was also investigated. The results, as shown in Fig. 2, indicate that the addition of a little amount of D-alanine to the medium containing 50 μg. of L-alanine causes better growth than that of the addition of L-alanine to the medium containing 50 μg. of D-isomer.

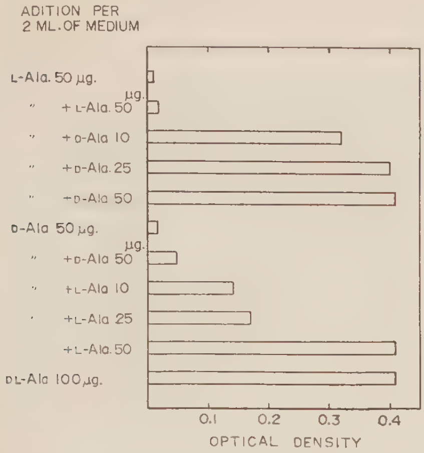

FIG. 2. Comparison of growth-promoting effect of alanine isomers for *Str. faecalis R* in the absence of vitamin B_6.

Incubation was carried out for 24 hours at 37°C.

Comparative Growth Response of Str. faecalis R to Vitamin B_6 Compounds in Alanine-Free medium—Experiment on the comparative growth response of *Str. faecalis R* to vitamin B_6 compounds revealed that growth of the organism increased in proportion to the amount of pyridoxine, pyridoxal or pyridoxamine added, as illustrated in Fig. 3. Pyridoxal was about 10,000 times, pyridoxamine about 5,000 times, as effective in promoting growth of this organism as was pyridoxine. Previously Snell and Rannefeld (*2*) reported that pyridoxamine was about 8,000 times, pyridoxal about 5,500 times, as active in promoting growth as was pyridoxine in the medium containing casein hydrolyzate, *i.e.*, in the presence of L-alanine.

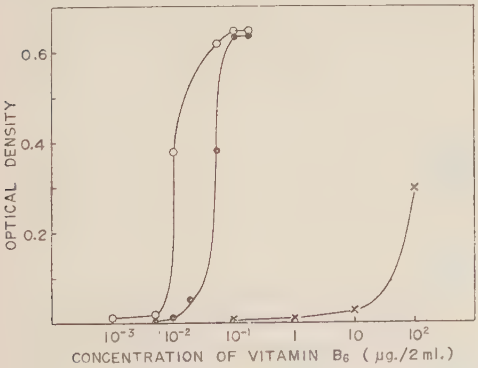

FIG. 3. Effect of vitamin B_6 compounds for the growth of *Str. faecalis R* in the absence of alanine.

Incubation was carried out for 24 hours at 37°C. —○— pyridoxal, —●— pyridoxamine, —×— pyridoxine

Growth Response of Leuc. mesenteroides P-60 to Alanine on Vitamin B_6-Free Medium—The growth response of *Leuc. mesenteroides P-60* to L-, D- and DL-alanines in the absence of vitamin B_6 was studied.

As indicated in Fig. 4, *Leuc. mesenteroides P-60* could not grow in the absence of alanine and the growth occurred by the addition of DL-alanine. That is, either alanine or vitamin B_6 is required for the growth of this organism. The addition of 2,000 μg. of DL-alanine to 2 ml. of the medium, gave the same final level of growth as that in the medium containing vitamin B_6. L-Alanine showed the same activity as DL-alanine for the growth of this organism, but D-alanine had only negligible activity.

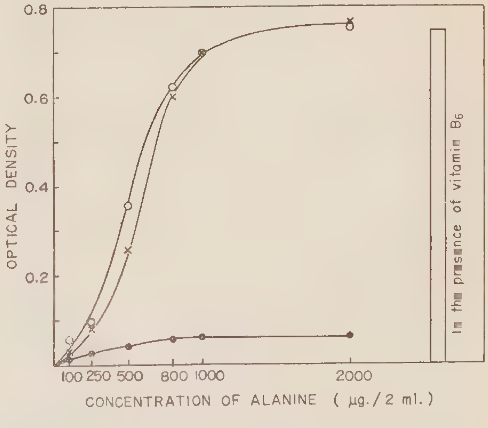

FIG. 4. Growth response of *Leuc. mesenteroides P-60* to alanine isomers in the absence of vitamin B_6.

Incubation was carried out for 24 hours at 37°C. —○— L-alanine, —●— D-alanine, —×— DL-alanine

Comparative Growth Response of Leuc. mesenteroides P-60 to Vitamin B_6 Compounds in Alanine-Free Medium—In alanine-free medium the growth of *Leuc. mesenteroides P-60* increased in response to the amount of pyridoxal or pyridoxamine added. Details of the results concerning the activities of the vitamin B$_6$ compounds for this organism are given in Fig. 5. In promoting growth pyridoxal was as about 1,000 times active as pyridoxamine, while the pyridoxine did not support the growth of *Leuc. mesenteroides* under these conditions.

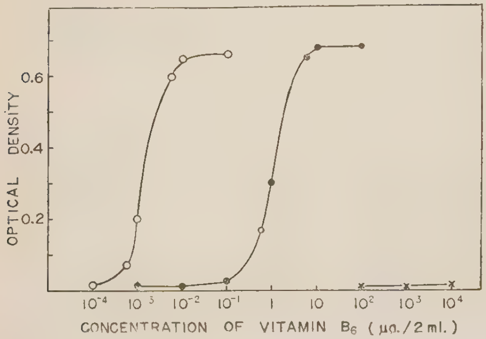

Fig. 5. Effect of vitamin B$_6$ compounds for the growth of *Leuc. mesenteroides P-60* in the absence of alanine.

Incubation was carried out for 24 hours at 37°C. —○— pyridoxal, —●— pyridoxamine, —×— pyridoxine

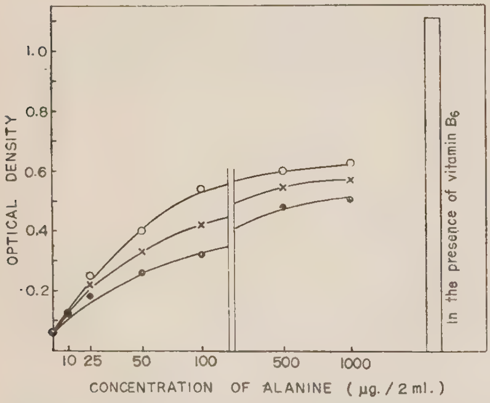

Fig. 6. Growth response of *Lact. arabinosus 17-5* to alanine isomers in the absence of vitamin B$_6$.

Incubation was carried out for 24 hours at 37°C. —○— L-alanine, —●— D-alanine, —×— DL-alanine

Growth Response of Lact. arabinosus 17-5 to Alanine on Vitamin B$_6$-Free Medium—The growth response of *Lact. arabinosus 17-5* to L-alanine, D-alanine and DL-alanine in the absence of vitamin B$_6$ is illustrated in Fig. 6. It is of interest to note that *Lact. arabinosus 17-5* grew slightly even in the absence of both alanine and vitamin B$_6$. This result is quite different from the report by Ogawa (*4*) that the growth of this organism was not observed in an alanine and vitamin B$_6$-free medium. The difference of the components of the media seems to account for the discrepancy between the results on the growth in the absence of alanine and vitamin B$_6$. In the vitamin B$_6$-free medium, the cell growth was increased in proportion to the amount of alanine added. A comparison of the effectiveness of the optical antipodes of alanine revealed that L-, D- and DL-alanines had approximately the same activity in promoting growth of this organism, although L-alanine was slightly more active than was D-alanine under these conditions. The level of full response obtained with vitamin B$_6$ could not be attained by the addition of alanine.

Comparative Growth Response of Lact. arabinosus 17-5 to Vitamin B$_6$ Compounds in Alanine-Free Medium—In the alanine-free medium, the growth was increased with the addition of pyridoxine, pyridoxal or pyridoxamine. Results concerning the activity of these vitamin B$_6$ compounds for the growth of this organism are given in Fig. 7. Pyridoxamine was about 100,000 times, pyridoxal about 10,000 times as

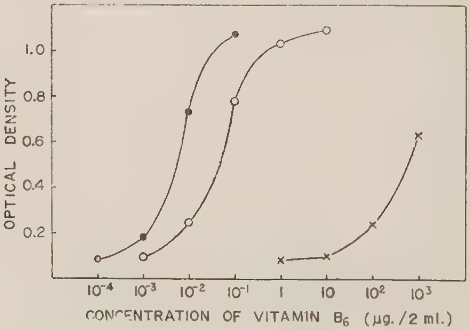

Fig. 7. Effect of vitamin B$_6$ compounds for the growth of *Lact. arabinosus 17-5* in the absence of alanine.

Incubation was carried out for 24 hours at 37°C. —○— pyridoxal, —●— pyridoxamine, —×— pyridoxine active in promoting growth as was pyridoxine. These results essentially coincide with the previous reports (*2*, *4*).

DISCUSSION

Although the growth of *Str. faecalis R* in the vitamin B_6-free medium supplemented with either L- or D-alanine alone was poor, the same level of full growth was attained by the addition of the both optical antipodes of alanine as in the presence of vitamin B_6. This fact suggests that the both isomers are required for this organism in the absence of vitamin B_6 and that the isomers can be synthesized when the vitamin is present in the medium.

The results, illustrated in Fig. 2, also indicate that more amount of L-alanine is required for the growth of *Str. faecalis R* than that of D-alanine. Previously Snell (*1*) reported that D-alanine is more active than L-alanine in the vitamin B_6-free medium. It is assumed that the effect of L-alanine was not clarified by the experiment by Snell, because he made the experiment in the medium with casein hydrolyzate containing L-alanine. Consequently, there is no essential contradiction between the results.

Difference observed in the ratio of activities of vitamin B_6 compounds for growth of *Str. faecalis R* is probably due to the difference of the composition of the media employed and of the amount of vitamin B_6 compounds added, as previously pointed out by Rabinowitz and Snell (*7*).

Leuc. mesenteroides P-60 grew well either in the alanine-free medium supplemented with vitamin B_6 or in the vitamin B_6-free medium supplemented with L- and DL-alanines. It is of interest to note that although D-alanine is not utilized, approximately the same response is obtained with L-alanine and DL-alanine.

By the present experiments, *Leuc. mesenteroides P-60* is found to require vitamin B_6 in the alanine-free medium. On the other hand, Dunn *et al.* (*8*), who used the medium containing DL-alanine, reported that vitamin B_6 is not essential for the growth of this organism. Accordingly, requirement of an organism for certain substance may have significance only in relation to the composition of the basal medium.

Lact. arabinosus 17-5 could grow slightly in the absence of both alanine and vitamin B_6, but the addition of L-, D- and DL-alanines showed marked acceleration of the growth. This finding indicates that the isomers of alanine are interconvertible in the organisms and utilized to stimulate the growth.

Three organisms used in the present experiments are capable to grow without alanine in the medium containing vitamin B_6. It is considered that the presence of vitamin B_6 enables the organisms to form necessary L-alanine and D-alanine by proper enzyme systems, in which vitamin B_6 compounds play an important rôle.

The behavior of *Lact. arabinosus 17-5* is different from that of *Str. faecalis R* and *Leuc. mesenteroides P-60*, *i.e.*, the maximum level of growth of *Lact. arabinosus 17-5* in the presence of vitamin B_6 is better than that in the presence of DL-alanine. This indicates that vitamin B_6 stimulates the growth of this organism also in other systems as well as in the system of alanine formation.

SUMMARY

The relationship of alanine and vitamin B_6 to growth of *Str. faecalis R*, *Leuc. mesenteroides P-60* and *Lact. arabinosus 17-5* was studied by employing chemically defined synthetic media. The results are as follows.

1. For the growth of *Str. faecalis R*, either alanine or vitamin B_6 was required. The growth of this organism was not measurably promoted by the addition of one optical antipode of alanine to the vitamin B_6-free medium, but the same final level of growth was attained by the addition of the both isomers as that of vitamin B_6.

2. *Leuc. mesenteroides P-60* required either alanine or vitamin B_6 for growth. In the vitamin B_6-free medium, the addition of L- or DL-alanine gave the same full growth as that in the presence of vitamin B_6, although the addition of D-alanine showed no effect for the growth of this organism.

Although slight growth of *Lact. arabinosus* was observed even in the absence of both alanine and vitamin B_6, the growth was markedly stimulated by the addition of alanine and vitamin B_6. The effectiveness of L-, D- and DL-alanines in promoting growth was at the same magnitude but the effect was inferior to the addition of vitamin B_6.

3. Of the vitamin B_6 compounds, the most active form in promoting growth was pyridoxamine for *Lact. arabinosus 17-5* and pyridoxal for *Str. faecalis R* and *Leuc. mesenteroides P-60*. Pyridoxine showed less or no activity in supporting growth of the lactic acid bacteria.

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Complexes between Acidic and Basic Proteins

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It has been known that mixing of acidic and basic proteins in solution results in the mutual precipitation of these proteins at the pH values intermediate to their isoelectric points. For example, a strongly basic protein of protamine (*1*) or lysozyme (*2*) forms precipitates with various acidic proteins, and the phenomenon has been used for the separation or purification of proteins without due physico-chemical investigation of the mechanism or the precipitates formed. Modern analytical techniques for macromolecules were recently applied by several workers for the study of this class of precipitation or complex formation, which is caused mainly by the electrostatic interactions between the oppositely charged protein molecules at the intermediate pH's. Steiner (*3*) observed the turbidity of serum albumin-lysozyme mixtures as a function of pH, ionic strength and protein concentration, and accounted for the light-scattering properties by his model of reversible complexing by electrostatic interaction. In fact, his ultracentrifugal analysis at a certain experimental condition showed a sedimentation peak which he interpreted as the peak of a soluble complex formed between those protein molecules. An electrophoretic study was made for the mixtures of serum albumin and thymus nucleic acid by Goldwasser and Putnam (*4*), who attributed the changes in mobility and in area of electrophoretic peaks to the formation of complexes.

The present paper deals with a similar line of study by ultracentrifugal and spectroscopic analyses for the mixtures of four different combinations between acidic proteins of α-casein and catalase and basic proteins of lysozyme and protamine, but considerably more detailed informations were obtained as to the make-up of their soluble and insoluble complexes as well as the direct evidence for complexing. In this report, the molar ratio of a basic protein to an acidic protein in a complex is designated as "association ratio" and is expressed by R to distinguish it from the molar ratio of the proteins dissolved to prepare the mixture.

EXPERIMENTAL

Materials—Pure α-casein was prepared from cow's skim milk by the method of Warner (*5*). Both electrophoretic and ultracentrifugal patterns of the preparation showed a single peak of α-casein. Crystalline catalase was prepared from bovine liver by the Shirakawa method (*6*), using ammonium sulfate for the separation and then recrystallizing several times by changing the pH of the solution. Lysozyme crystals were obtained from hen's egg white according to the method of Alderton and Fevold (*7*). Protamine (clupein) sulfate was purchased from Tokyo Industrial Co. and was a recrystallized pure sample. These proteins were first dissolved in water and then the solution was adjusted to a given pH and ionic strength(μ) with KH_2PO_4-HCl or Na_2HPO_4-KH_2PO_4 buffer. The molar concentrations of α-casein, lysozyme and protamine were determined gravimetrically, assuming their molecular weights to be 121,800 (*8*), 14,700 (*9*) and 6,000 (*10*), respectively. The concentration of catalase was determined spectroscopically with aid of its molar extinction coefficient(ε) of 3.4×10^5 at 406 mμ (*11*).

Ultracentrifugal Analysis—Analytical ultracentrifugations were carried out at 59,780 r.p.m. ($260,000\times g$) in a Spinco Ultracentrifuge, Model E. The partial specific volume assumed for the calculation of sedimentation coefficients ($S_{w,\ 20}$) in water at 20°C) was 0.724, 0.730, 0.750, 0.750 for α-casein (*12*), catalase (*13*), lysozyme (*14*) and protamine, respectively. For the calculation of $S_{w,\,20}$ for a complex between these proteins, the partial specific volume for the larger component was used. Sedimentation constants at zero protein concentration were estimated by extrapolation from the $S_{w,\,20}$ values at various protein concentrations. The constants determined at pH 7.0 for these proteins were as follows; α-casein, 3.9 ± 0.3 *S* at $\mu = 0.306$, catalase, 10.9 ± 0.2 *S* at $\mu = 0.055$, lysozyme, 1.9 ± 0.2 *S* at $\mu = 0.055$, and protamine, 0.8 ± 0.2 *S* at $\mu = 0.110$. These values agree approximately with the values obtained at the similar experimental conditions by various workers (*8*, *9*, *15–17*).

RESULTS

1. *Process of Complexing*—When a solution of lysozyme was added to an α-casein solution, precipitates were formed under certain conditions of pH, ionic strength(μ) and protein concentration. As a preliminary test, the effects of pH and ionic strength upon the formation of precipitates were observed at 17–19°C for the mixture of 4.1×10^{-5} *M* α-casein and 15.6×10^{-5} *M* lysozyme. The results are listed in Table I, where the marks (+) and (−) indicate formation and non-formation of precipitates, respectively. The mark, (±) was written for the results that the mixture was transparent at 17–19°C but became turbid when warmed up by several degrees. The mixtures at such an intermediate condition were suitable for the observation of the sedimentation peak of a soluble complex between these proteins because of no loss of the complex by precipitation. The first row in Table I shows that such a condition is obtainable when $\mu = 0.304$ at pH 7.0. The change of pH at this value of ionic strength effected precipitation between pH 4.0 and 6.3, the range being intermediate to the isoelectric points, 4.0 and 11.0 of α-casein and lysozyme, respectively.

Ultracentrifugal analyses were carried out at pH 7.0 and $\mu = 0.304$ for α-casein-lysozyme mixtures, in which lysozyme concentration was varied from zero to 24.0×10^{-5} *M* and α-casein concentration was fixed at 4.1×10^{-5} *M*. Two peaks were generally observed in the sedimentation patterns. The coefficient for the lighter component agreed with the value, 1.9 *S* for pure lysozyme solution and showed no variation with the increase of lysozyme concentration. The coefficients for the heavier component are plotted against lysozyme concentration in Fig. 1. The coefficient below 15.1×10^{-5} *M* lysozyme agreed approximately with the value, 4.6 *S* for pure α-casein solution free from lysozyme at the same pH and μ. The horizontal line in the figure is drawn on the level of 4.7 *S* which is the average of the coefficients observed below the lysozyme concentration. When the concentration was raised to 15.6×10^{-5} *M*, both α-casein and lysozyme peaks were considerably lowered

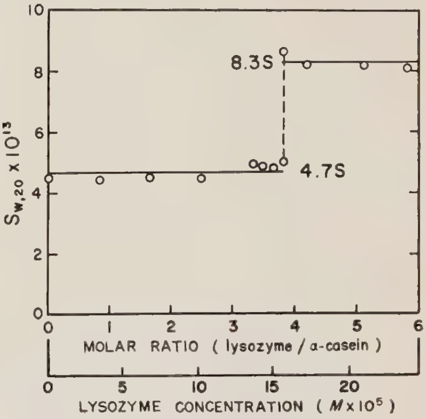

Fig. 1. Association of α-casein with lysozyme; the concentration of α-casein, 4.1×10^{-5} *M*, pH 7.0, and $\mu = 0.304$.

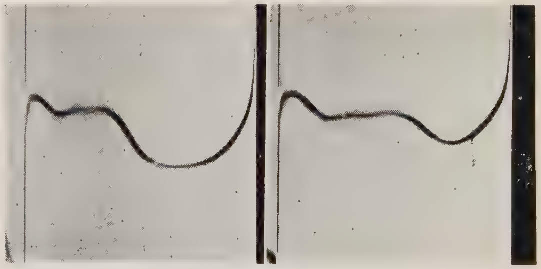

Fig. 2. Sedimentation diagram of α-casein-lysozyme mixture in phosphate buffer; pH 7.0 and $\mu = 0.304$. The final concentrations of α-casein and lysozyme are 9.1×10^{-5} *M* and 17.6×10^{-5} *M* respectively. $S_{w,\,20}$ values for the heavier, the middle and the lighter component are 8.5 *S*, 4.6 *S* and 1.8 *S* respectively. (a) At 32 minutes after reaching the maximal speed of 59,780 r.p.m.; bar angle 40°. (b) At 40 minutes after reaching the maximal speed.

Table I

The Effect of pH and Ionic Strength on the Formation of Precipitates in the Mixtures of Acidic and Basic Proteins

Sample and its concentration in 10^{-5} M	pH or μ	Precipitation				
α-Casein 4.1	μ at pH 7.0	0–0.302 +	0.304 ±	0.306 −		
Lysozyme 15.6	pH at $\mu=0.304$	2.0 −	2.6 ±	4.0–6.3 +	7.0–8.3 ±	9.0 −
α-Casein 4.1	μ at pH 7.0	0–1.505 +				
Protamine 38.4	pH at $\mu=0.753$	3.0–10.3 +	11.0 ±	11.8 −		
Catalase 2.0	μ at pH 7.0	0–0.037 +	0.055 ±	0.110–0.420 −		
Lysozyme 15.6	pH at $\mu=0.055$	3.8 −	4.3 ±	5.0–6.4 +	7.0–10.1 ±	11.1 −
Catalase 2.0	μ at pH 7.0	0–0.110 +	0.121–0.220 −			
Protamine 38.4	pH at $\mu=0.110$	4.0 ±	5.4–12.0 +	12.7 ±		

and a new peak of 8.3 *S* appeared (See Fig. 2). The lowering of the peaks bears evidence that the 8.3 *S* component is the soluble complex between these proteins. The mixtures with more concentrated lysozyme showed the peaks of lysozyme and the 8.3 *S* component but no peak of 4.7 *S* of α-casein, and precipitates were formed above 20.0×10^{-5} M lysozyme. The higher concentration required for the formation of precipitates as compared with that for the 8.3 *S* soluble complex is an indication that the precipitates were formed through the soluble complex as the intermediate. The mechanism of the transformation of the soluble complex into precipitates will be discussed in the later section where the association ratios for the soluble complex and the precipitates are presented. When NaCl (final concentration, 1.0 *M*) was added to the mixtures containing the soluble complex, the 8.3 *S* peak disappeared and a peak of 5.8 *S* appeared. The value, 5.8 *S* agreed with the coefficient observed for α-casein free from lysozyme in the same medium containing NaCl and buffer. This result proved the reversibility of the complexing process.

Protamine is more effective than lysozyme to obtain precipitates with α-casein. The result obtained in qualitative precipitation test with 4.1×10^{-5} M α-casein and 38.4×10^{-5} M protamine is shown in Table I. At pH 7.0, the mixture showed precipitates over the entire range of ionic strength observed. The change of pH at $\mu=0.753$ showed precipitates at pH values below 11.0. No precipitation above the pH value may be interpreted as being due to the reduction of electrostatic attraction at the pH value close to the isoelectric point of protamine and/or the indirect effect of the dissociation of α-casein into its subunits which was observed by von Hippel and Waugh (*15*).

Ultracentrifugal analyses were made for the mixtures of 4.1×10^{-5} M α-casein and $(0\text{–}49.3) \times 10^{-5}$ M protamine at pH 7.0 and $\mu=0.753$. Similarly as above, two peaks were observed in the sedimentation patterns. The peak of the lighter component of 0.8 *S* may be considered as that of free protamine, and its coefficient did not vary over the range of protamine concentration. The coefficients observed for the heavier component are shown in Fig. 3 as a function of protamine concentration. The values below $23.0\times10^{-5}\,M$ protamine were agreed with the value, 5.2 S for α-casein free from protamine at the same pH and μ. The coefficient was higher and reached the level of 7.1 S above this concentration, where a considerable amount of precipitates was formed. When NaCl (final concentration, 1.0 M) was added to the mixture at the critical protamine concentration of $33.0\times10^{-5}\,M$, the 7.1 S peak disappeared and the peak of free α-casein appeared, so that the complexing is a reversible process.

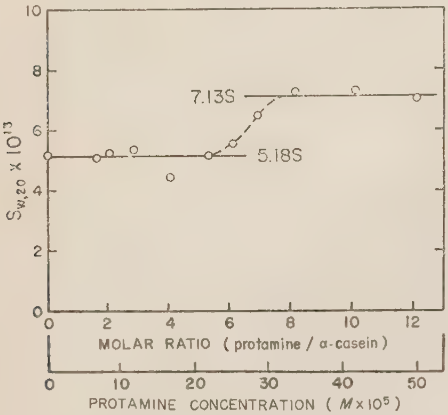

Fig. 3. Association of α-casein with protamine; the concentration of α-casein, $4.1\times10^{-5}\,M$, pH 7.0, and $\mu=0.753$.

The similar phenomenon of forming a soluble complex was observed when α-casein as the acidic component was replaced by catalase. Precipitation tests with $2.0\times10^{-5}\,M$ catalase and $15.6\times10^{-5}\,M$ lysozyme or $38.4\times10^{-5}\,M$ protamine showed that the critical ionic strength to obtain precipitates at pH 7.0 was 0.037–0.055 with lysozyme and 0.110–0.121 with protamine (See Table I). These values are lower than the corresponding values obtained with α-casein as the acidic component, so that the catalase molecule interacts more weakly with the basic proteins than does the α-casein molecule. The pH range to obtain precipitates (+) or the soluble complex (±) at the critical ionic strength was 4.3–10.1 for the catalase-lysozyme mixture and 4.0–12.7 for the catalase-protamine mixture, the lower and upper limits of the ranges being in rough agreement with the isoelectric points of the component proteins.

The sedimentation coefficient for the heavier component in the mixtures of $2.0\times10^{-5}\,M$ catalase and $(0\text{–}54.5)\times10^{-5}\,M$ lysozyme at pH 7.0 and $\mu=0.055$ are shown in Fig. 4, which indicates an abrupt increase of the coefficient from 10.9 to 12.0 S between lysozyme concentrations, 7×10^{-5} and $11\times10^{-5}\,M$. The area of the lysozyme peak for the mixture containing the 12.0 S component was lower than that observed for the same concentration of lysozyme solution free from catalase, the fact being the evidence that the 12.0 S component is a soluble complex formed between lysozyme and catalase (See Fig. 5). Fig. 6 shows the coefficients for the heavier component in the mixtures of $2.0\times10^{-5}\,M$ catalase and $(0\text{–}236)\times10^{-5}\,M$ protamine at pH 7.0 and $\mu=0.110$. A rather high protamine concentration more than $50\times10^{-5}\,M$ was required to obtain an appreciable change of the coefficient. Above $80\times10^{-5}\,M$ prota-

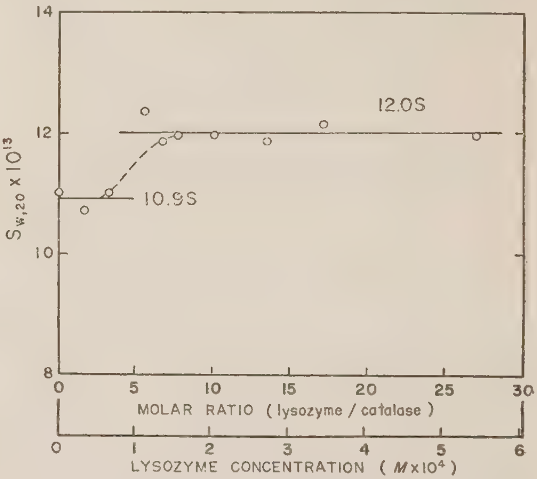

Fig. 4. Association of catalase with lysozyme; the concentration of catalase, $2.0\times10^{-5}\,M$, pH 7.0, and $\mu=0.055$.

mine, the coefficient reached a constant level of 11.8 *S*, indicating the formation of a soluble complex.

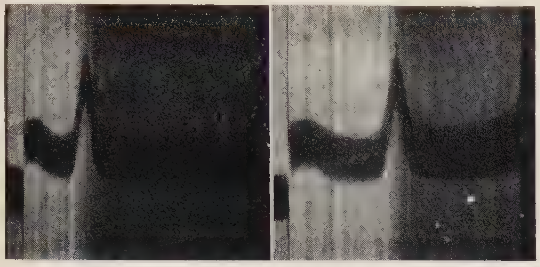

FIG. 5. Sedimentation diagram of catalase-lysozyme mixture in phosphate buffer; pH 7.0 and $\mu=0.055$. The final concentrations of catalase and lysozyme are $2.0\times10^{-5}\,M$ and $15.6\times10^{-5}\,M$ respectively. $S_{w,20}$ values for the heavier and the lighter component are 12.0 *S* and 2.0 *S* respectively. (a) At 17 minutes after reaching the maximal speed of 59,780 r.p.m.; bar angle 70°. (b) At 33 minutes after reaching the maximal speed; bar angle 65°.

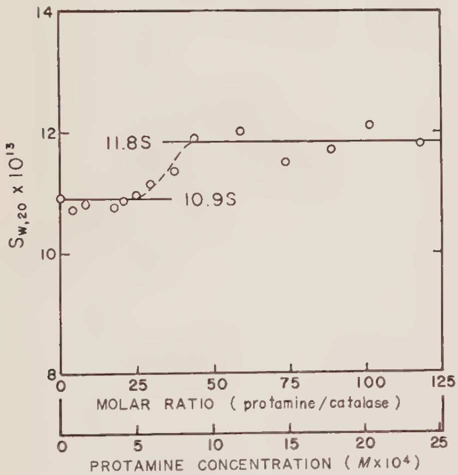

FIG. 6. Association of catalase with protamine; the concentration of catalase, $2.0\times10^{-5}\,M$, pH 7.0, and $\mu=0.110$.

Catalase activity was measured at pH 7.0 by the Euler and Josephson method (*18*) for the mixtures containing the soluble complex with lysozyme or protamine. In the measurements, catalase concentration was kept constant at $7.0\times10^{-9}\,M$, and the activity of the same concentration of catalase solution free from the basic proteins was taken as unity. The relative activity thus measured was 35 per cent with $20.0\times10^{-5}\,M$ lysozyme at $\mu=0.055$ and was 81 per cent with $118\times10^{-5}\,M$ protamine at $\mu=0.110$. These concentrations chosen for lysozyme and protamine are the minimum concentrations to observe the complete transformation of free catalase into its soluble complexes by sedimentation analysis, although the catalase concentration in the activity measurement is much lower than that in the sedimentation analysis. This result shows that the catalase molecule possesses a considerable activity even in its associated state with the basic proteins. The higher activity observed for the catalase-protamine complex may have some connection with the smaller molecular weight of protamine, which may have less chance of masking the heme groups of catalase, and vice versa. The low concentration of catalase to be used for activity measurements in contrast with the high concentration for sedimentation analyses unable us to obtain the more detailed correlation between activity and sedimentation coefficient.

2. *Association Ratio for Soluble Complexes and Precipitates*—The molar association ratio of lysozyme to α-casein or catalase for their soluble complex was determined based upon the principle, which is explained below for α-casein-lysozyme system. The area, *A* of the lysozyme peak for a mixture containing the 8.3 *S* soluble complex but no precipitate was appreciably smaller than the area, A_0 of the peak for the same concentration of lysozyme solution free from α-casein. It is therefore, evident that the fraction of lysozyme corresponding to $(A_0-A)/A_0$ formed the soluble complex with α-casein. Since the total molar concentration, L_0 and C_0 of lysozyme and α-casein used for the preparation of the mixture are known and no free α-casein remains in the mixture, we can evaluate the association ratio, *R* of lysozyme to α-casein for the soluble complex, which is equal to $L_0\,(A_0-A)/C_0A_0$. Repeated measurements of this ratio gave us the value of 2.4 ± 0.3 for the mixtures of $4.1\times10^{-5}\,M$ α-casein and $(15.6–17.0)\times10^{-5}\,M$ lysozyme at pH 7.0 and $\mu=0.304$, in which

no precipitate was formed. The association ratio of lysozyme to catalase for the 12.0 *S* soluble complex in the mixtures of 2.0×10^{-5} *M* catalase and $(6.4–12.7)\times10^{-5}$ *M* lysozyme at pH 7.0 and $\mu=0.055$ was observed similarly and was found to be 3.3±0.4 (See Table III a). The association ratios for the soluble complexes with protamine could not be determined because of a large error included in the measurement of the area of the protamine peak with slow sedimentation rate.

The association ratios for the precipitates formed at pH 7.0 and the same ionic strengths as used for the observation of the *R* values of soluble complexes were determined spectroscopically by the procedure explained below for α-casein-lysozyme system. A precipitate suspension prepared with $(2.42–2.65)\times10^{-5}$ *M* α-casein and $(35.4–60.0)\times10^{-5}$ *M* lysozyme at pH 7.0 and $\mu=0.304$ was centrifuged to remove the precipitates. To the supernatant containing the soluble complex and excess free lysozyme, NaCl was added to make the soluble complex completely dissociated into its component proteins. The solution thus obtained was then diluted appropriately with the buffer of pH 7.0 and $\mu=1.100$. The absorbance value(E) of this diluted solution was observed at two different wavelengths of 250 and 280 mμ, and the concentrations of lysozyme and α-casein in the supernatant before dilution were calculated from the absorbance values with aid of the molar extinction coefficients(ε) for the component proteins listed in Table II. Since the molar concentrations of α-casein and lysozyme used for the preparation of the precipitates are known, one may calculate the association ratio for the precipitates from their molar concentrations in the supernatant and its volume.

TABLE II

The Molar Extinction Coefficients(ε) Used for the Determination of Association Ratios

Sample	ε_{250}	ε_{280}	ε_{310}	ε_{406}
α-Casein	7.26×10^4	1.33×10^5	—	—
Lysozyme	2.11×10^4	4.47×10^4	—	—
α-Casein	7.26×10^4	1.33×10^5	—	—
Protamine	3.70×10^2	2.37×10^2	—	—
Catalase	—	—	8.84×10^4	3.40×10^5
Lysozyme	—	—	6.43×10^2	1.86×10^2
Catalase	—	—	8.84×10^4	3.40×10^5
Protamine	—	—	1.82×10^2	5.83×10

TABLE III

Association Ratios, R of Soluble Complexes and Precipitates at pH 7.0

The concentrations of proteins are expressed in 10^{-5} *M* units, and are those used for the determination of *R* values except for soluble complexes with protamine, for which the lower limit for complexing is listed.

(a) *Soluble Complexes*

Sample and its concentration in 10^{-5} *M*	Sedimentation coefficient	μ	*R*
α-Casein 4.10 Lysozyme 15.6–17.0	8.3 *S*	0.304	2.4±0.3
α-Casein 4.10 Protamine 23.0	7.1 *S*	0.753	—
Catalase 2.00 Lysozyme 6.40–12.7	12.0 *S*	0.055	3.3±0.4
Catalase 2.00 Protamine 80.0	11.8 *S*	0.110	—

(b) *Precipitates*

Sample and concentration in 10^{-5} *M*	Before water treatment		After water treatment	
	μ	*R*	μ	*R*
α-Casein 2.42–2.65 Lysozyme 35.4–60.0	0.304	6±1	0	27±3
α-Casein 0.87–3.11 Protamine 107–131	0.753	8±1	0	43±6
Catalase 6.75 Lysozyme 54.5–68.0	0.055	8±2	0	38±6
Catalase 2.02 Protamine 154–241	0.110	27±5	0	71±5

The second column in Table IIIb shows

the association ratios determined for various precipitates formed between the basic and acidic proteins at the same ionic strengths as used for the determination of the *R* values of soluble complexes, and Table II includes the wavelengths chosen for the determinations and the molar extinction coefficients assumed at the wavelengths for the component proteins. It is clear from the result that more molecules of basic protein are required for the formation of precipitates from the soluble complexes. In other words, the process of precipitation is not merely the polymerization of the soluble complex molecules, and involves the association of the soluble complexes with additional molecules of basic proteins.

Similar spectroscopic observations were made directly for the solution of the precipitates separated from the supernatant by decantation. The measurements, however, showed no reproducible result which seemed to be due to the residual supernatant on the precipitates. Trials to remove it by washing the precipitates with the buffer of the same pH and ionic strength were unsuccessful because the addition of a small volume of the buffer completely dissolved the precipitates.

It was found in the above experiment that almost all of the precipitates remained undissolved when washed with distilled water. The spectroscopic measurements of the extracts, however, showed that water added not only removed the residual supernatant but also dissolved the precipitates slightly. In fact, the association ratio observed similarly as above for the water-treated precipitates increased in each time of treatment, and reached a constant value after 10–15 times of treatment. The values of *R* thus obtained after the sufficient times of water treatment are listed in the third column in Table IIIb, and may be regarded as the association ratios at $\mu=0$. Much greater values of *R* thus obtained as compared with the values before treatment indicate the great dependency of the electrostatic attraction between basic and acidic proteins upon the ionic strength of the medium, which is reflected also in the results of the qualitative precipitation tests shown in Table I.

DISCUSSION

The sedimentation coefficients and association ratios observed for various soluble complexes and precipitates are summarized in Table III. The value, 8.3 *S* for the α-casein-lysozyme complex as compared with 4.7 *S* for the native α-casein molecule suggests that the complex is either monomer or dimer with respect to the number of the α-casein molecules in the complex, unless the complex is a largely streched or unfolded polymer of α-casein. According to the observation of Sullivan, Fitzpatric, Stanton, Annino, Kissel and Palermiti (*8*), the α-casein molecule at neutral pH is approximately globular, having molecular weight of 121,800. The calculation based on this molecular weight and the coefficient, 4.7 *S* obtained in this study gave us the value of 1.92 as its frictional ratio, which indicates that the molecule is slightly elongated in the solvent medium used. If the soluble complex contains one molecule of α-casein, the molecular weight of the complex is $121{,}800+(2\times14{,}700)=151{,}200$, since the association ratio is approximately 2. The further theoretical calculation based on this molecular weight showed that the coefficient for the complex would be 10.4 *S* if the complex is completely spherical, and would be 5.4 *S* if the frictional ratio is the same as that of the α-casein molecule of 4.7 *S*. The observed value of 8.3 *S* is in between these calculated values. The similar calculation based upon the dimer hypothesis with molecular weight of 302,400 showed its coefficient of 16.6 *S* for a spherical shape and 8.6 *S* for the same frictional ratio as that for the native α-casein molecure. These calculated coefficients could eliminate neither the monomer or dimer hypothesis. The coefficient, 7.1 *S* for the complex between α-casein and protamine is lower than 8.3 *S* for the α-casein-lysozyme complex. The lower value may be at least partially due to the smaller molecular weight of protamine.

The gradual increase of the coefficient from 5.2 to 7.1 *S* at the intermediate stage of complex formation may be an indication of the formation of complexes carrying a smaller number of protamine molecules.

The small increments by complexing of sedimentation coefficient of catalase indicate undoubtedly that one molecule of catalase is involved in the complex formation. The association ratio determined for the soluble complex between catalase and lysozyme was approximately 3, so that the molecular weight of the complex would be $248{,}000+(3\times 14{,}700) = 292{,}100$. The calculation with this molecular weight and the same frictional ratio, 1.29 as that of the native catalase molecule of 10.9 *S* gave us the value of 12.2 *S* as the coefficient of the complex, which is close to the observed value of 12.0 *S*. The coefficient, 11.8 *S* for the catalase-protamine complex is also similar to this calculated value. The similarity despite the smaller molecular weight of protamine as compared with that of lysozyme suggests that the association ratio for the catlase-protamine complex is greater than 3 obtained for the catalase-lysozyme complex.

As is clear from the association ratios summarized in Table III, the *R* value for the precipitates with protamine is higher than the corresponding value for the precipitates with lysozyme. This fact is, probably, due to both smaller molecular weight and stronger basic character of protamine as compared with those of lysozyme. Judging from the results of the precipitation tests, the catalase molecule seems to interact more weakly with a basic protein than does the α-casein molecule, whereas the association ratio for a soluble complex or precipitates of catalase with a basic protein is greathr than the value for those of α-casein with the same basic protein. The stronger interaction observed with α-casein may be due to the more acidic character of α-casein, and the larger association ratio may arise from the larger molecular weight of catalase, and vice versa. These different properties of association ratio and the strength of interaction have to be distinguished clearly and to be discussed separately in the future study of the mutual complexing between acidic and basic proteins.

SUMMARY

The mutual complexing between four different combinations of acidic proteins of α-casein and catalase and basic proteins of lysozyme and protamine was studied by both ultracentrifugal and spectroscopic analyses. It was found from the analyses that the molecule of the acidic proteins forms a soluble complex with a few molecules of the basic proteins and the further increase of basic protein concentration results in a mutual precipitation. The formation of precipitates was found to be not merely the polymerization of the soluble complex molecules, and involves the association of the complex with additional molecules of basic proteins. The molar association ratios of basic proteins to acidic proteins were measured for the soluble complexes and precipitates, and the results were discussed in relation to the acidic or basic characters and the molecular weights of the component proteins.

The author wishes to express his sincere gratitude to Prof. K. Shibata for his kind guidance and valuable advices in carrying out the experiments described here, and to Dr. J. Hirano for his advices in preparing this manuscript. He is also grateful to Mrs. M. Kamata for her assistance in this study.

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The Journal of Biochemistry, Vol. 49, No. 5, 1961

Fate of Some Sedatives in the Animal Body

I. α-Bromoisovalerylurea (Bromural)

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(Received for publication, December 20, 1960)

It is well known that the administration of organic halogenated compounds to animals causes urinary excretion of their cysteine conjugates. There are some sedatives having the structure of halogenated fatty-acylurea, but their metabolic fate is not exactly known.

The author has recently found that the administration of Bromural to some persons causes urinaly excretion of a new amino acid, which was isolated and its chemical structure was established as S-(isopropyl-carboxymethyl)-cysteine, which had already been isolated from the urine of hypercholesterolemic patients and named isovalthine by Mizuhara *et al.* (*1–3*).

EXPERIMENTALS AND RESULTS

Preparation of Amino Acid Fractions from Human Urine

(*I*) *Normal Human Urine*—The urine was collected from three women under toluene. One liter of the urine and the same volume of concentrated hydrochloric acid were mixed and the mixture was heated on a sand bath for 20 hours. The hydrolysate was evaporated to dryness in vacuum and the excess hydrochloric acid was removed by repeated evaporation. The residue was taken up in water and the aqueous solution was adjusted to pH 6 by adding concentrated ammonia and filtered.

The ampholyte fraction in the filtrate was collected by using ion exchangers according to the method of Carsten (*4*). The ion exchangers used were H-from of Diaion SK-1 (cation exchanger, Mitsubishi Kasei Co., Ltd.) and OH-form of Diaion SA-100 (anion exchanger, Mitsubishi Kasei Co., Ltd.). The ampholyte fraction eluted from the anion exchanger was evaporated to dryness in vacuum and the excess hydrochloric acid was removed by repeated evaporation. The residue was taken up in water (about 500 ml.), the aqueous solution was adjusted to pH 5 with ammonia, and the humin-like substance that appeared was filtered off. The filtrate was passed through a column of Cl-form of Amberlite CG-45. Preparation of the Amberlite column was the same as described before (*2*).

The effluent from the Amberlite column was desalted by Diaion SA-100. The paper chromatogram of this fraction is shown in Fig. 1. Solvents used for paper chromato-

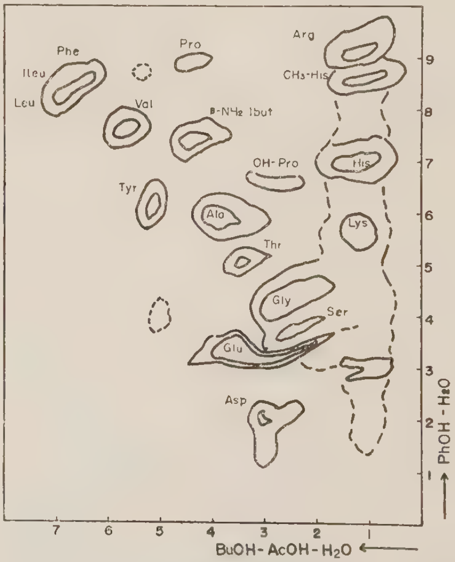

FIG. 1. Non-acidic amino acid fraction in the Normal human urine (before Bromural intake).

graphy were butanol-acetic acid-water (4 : 1 : 4 v/v, 0.2% NH_3 present in the cell) throughout the present work. The unadsorbed fraction contains mainly non-acidic amino acids.

The fraction adsorbed on the Amberlite column was eluted by *N* hydrochloric acid. The eluate containing acidic amino acids was evaporated to dryness in vacuum. The acidic amino acid pattern is shown in Fig. 2.

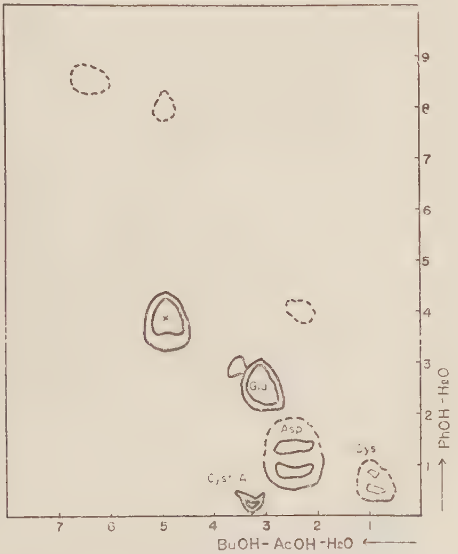

Fig. 2. Acidic amino acid fraction in the normal human urine (before Bromural intake).

Besides spots for glutamic, aspartic, and cysteic acids, an unknown spot (R_f value 0.49 and 0.39) was found on the paper chromatogram. The unknown spot was positive to sulfur tests on paper chromatogram (5, 6) and the concentrate of the spot, prepared by cellulose column fractionation, gave alanine by treatment with Raney nickel. The unknown amino acid ran on paper exactly as did the synthetic compound, *i.e.* S-(2-methyl-2-carboxyethyl) cysteine,* with the solvent systems of BuOH-AcOH-H_2O, pyridine-H_2O-conc.NH_3 (20 : 2 : 1 v/v) and pyridine-BuOH-H_2O (2 : 1 : 1 v/v).

Although the isolation of this amino acid has not been achieved, the amino acid is now tentatively named "Isobuteine." Isobuteine is found quite often in normal human urine.

(*II*) *Human Urine after Bromural Intake*—Bromural was administered to the three women for two days (total, 3 g. to each woman). Their urine was collected under toluene for three days. About ten liters of the urine was made alkaline by the addition of concentrated ammonia and the subseqnent precipitate was filtered off. The pH of the filtrate was adjusted to around 6 with hydrochloric acid. The weak acidic urine was passed through a column (10×60 cm.) of Diaion SK-1 (H-form). The effluent (Fraction A) was stored under toluene in a cold room.

The fraction adsorbed on Diaion SK-1 (Fraction B) was separated into the acidic and non-acidic fractions by the same method as described above in the experiment (I). In this case, hydrolysis of the Fraction B was carried out before treatment through the Amberlite column. The paper chromatograms of the acidic and non-acidic amino acid fractions are shown in Figs. 3 and 4, respectively.

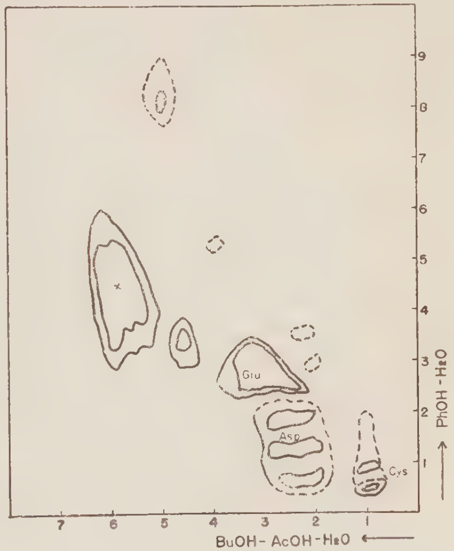

Fig. 3. N-free acidic amino acid fraction in the normal human urine (after Bromural intake).

A new large spot having higher R_f values (0.59 and 0.45) than that of isobuteine was seen in the acidic amino acid pattern (Fig. 3). As will be shown later, the new amino acid was identified as S-(isopropyl-carboxymethyl) cysteine (*1–3*).

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* Isolation and synthesis of isobuteine will be published in a separate paper.

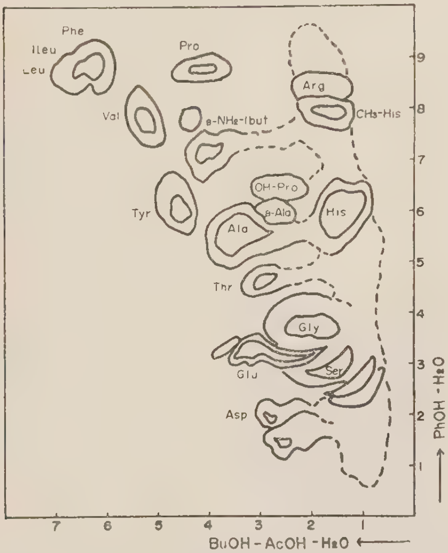

Fig. 4. N-free non-acidic amino acid fraction in the normal human urine (after Bromural intake).

The fraction A not adsorbed on Diaion SK-1 was concentrated to about one liter. One liter of concentrated hydrochloric acid was mixed into the urine concentrate and the mixture was heated on a sand bath for 20 hours. The hydrolysate was evaporated to dryness and the excess hydrochloric acid was removed by repeated evaporation. The residue was taken up in water and the ampholyte in the aqueous solution was separated into acidic and non-acidic amino acid fractions by the treatment with Diaion SK-1 and Amberlite CG-45 as described above. The treatment with Diaion SA-100 was omitted in this case.

The paper chromatograms of the acidic and non-acidic amino acid fractions are shown in Figs. 5 and 6, respectively. The largest spot (R_f 0.57 and 0.48) in the acid amino acid pattern was identical with isovalthine, as will be shown later.

The hydrolysate of the effluent from the Diaion SK-1 column thus contains isovalthine, indicating that some of isovalthine is excreted as the N-covered form in normal human urine after Bromural intake. However, in the urine of a patient of cretinism who had never taken Bromural, isovalthine was excreted only in the N-free form (*2*). The above fact may be a point of difference between normal and diseased persons.

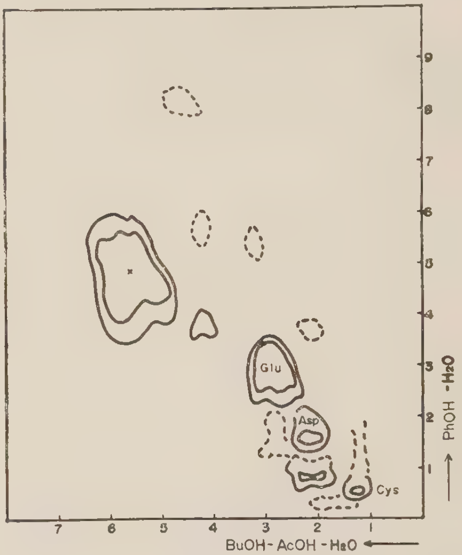

Fig. 5. N-covered acidic amino acid fraction in the normal human urine (after Bromural intake).

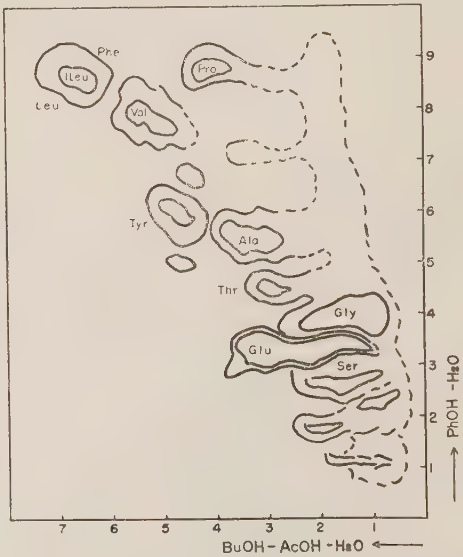

Fig. 6. N-covered non-acidic amino acid fraction in the normal human urine (after Bromural intake).

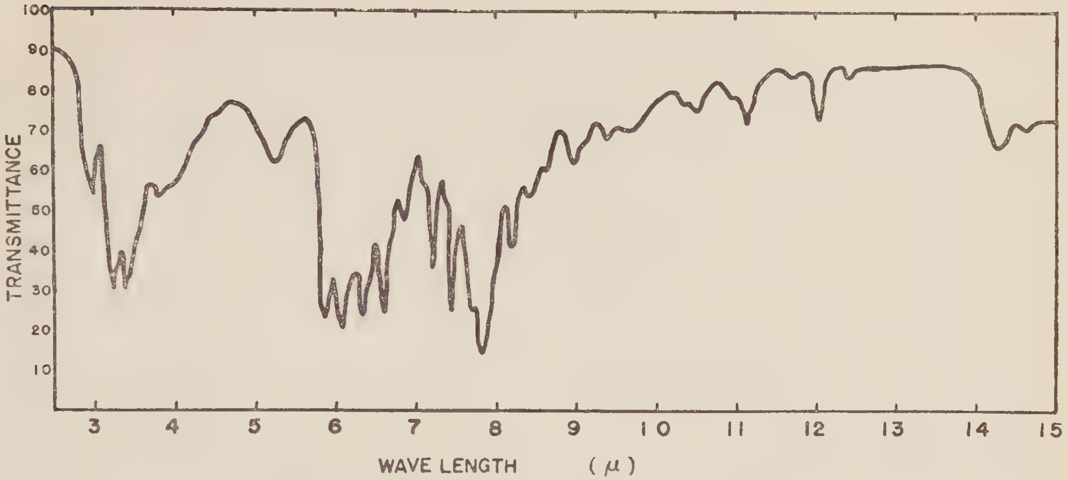

Fig. 7. Infrared spectrum of Bromural metabolite.

Isolation and Identification of Bromural Metabolite

The method of isolation of the Bromural metabolite is essentially the same as that of isovalthine (*2*). The acidic amino acids of Fractions A and B were combined and the mixture was fractionated by a cellulose column.

About 5 mg. of rhombic plates were obtained by repeated recrystallization from 50% ethanol. It decomposed at 213°C. Anal. Calcd. for $C_8H_{15}O_4NS$: C, 43.43; H, 6.78. Found: C, 43.65; H, 7.05. Its infrared spectrum (Fig. 7) was identical with that of isovalthine (*2*, *3*).

DISCUSSION

It has been proved that one of the metabolites of Bromural in the human body is isovalthine. Isovalthine had been isolated from the urine of hypercholesterolemic patients, such as those having myxoedema and atherosclerosis, and has also been found in the urine of cats and in patients of cretinism and severe diabetes. However, Bromural is often used as a sedative in hospitals and there is a possibility that these patients might have taken Bromural. Reexamination of the urine of patients with cretinism and atherosclerosis, who never took Bromural, has proved that these patients excrete isovalthine. Futhermore, administration of leucine to a cat causes a remarkable isovalthinuria.*

SUMMARY

α-Bromoisovalerylurea (Bromural) was administered to normal women and one of the metabolites isolated from the urine was identified as S-(isopropyl-carboxymethyl)-cys teine. The metabolite was excreted both in the N-free and N-covered forms.

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Studies in Lysine Biosynthesis

II. Metabolic Fate of DL-α-Aminoadipic Acid-6-C^{14} in *T. utilis*

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(Received for publication, January 5, 1961)

The formation of lysine from α-aminoadipic acid (α-AAA) with baker's yeast and *T. utilis* has been described in the previous paper (*1*), and the incorporation of acetate-1-C^{14} or C^{14}-glucose into protein lysine is depressed by addition of α-AAA as well as exogeneous lysine (*2*). Although this pathway was suggested to be of the first importance in the biosynthesis of lysine in yeast by Strassman and Weinhouse (*3*), no evidence has been obtained whether the metabolic fate of α-AAA leading to the production of lysine is only the way in yeast or not. For this reason, a study of the utilization of α-AAA-6-C^{14} was undertaken in an effort to clarify biosynthetic mechanisms operating in the cells of *T. utilis.*

The results presented in this paper indicate that the main route of α-AAA metabolism involves extensive conversion of its whole carbon chain to lysine, along with a small amount of radioactivity in two neutral amino acids, δ-aminovaleric acid and possibly α-hydroxy-ε-amino-hexanoic acid, suggesting catabolic pathways of lysine in *T. utilis.*

EXPERIMENTALS

Materials—DL-α-AAA-6-C^{14} was prepared from ethyl-α-acetamido-α-carbethoxy-δ-bromovalerate and $KC^{14}N$ *via* ethyl-α-acetamido-α-carbethoxy-δ-cyanovalerate by the method of Rothstein and Claus (*4*). The specific activity of α-AAA was 1.59×10^4 c.p.m. per μmole. DL-α-AAA, DL-α-hydroxy-ε-aminohexanoic acid, DL-α-amino-ε-hydroxyhexanoic acid, and DL-piperidine-2-carboxylic acid were the same as those described previously (*1*). δ-Aminovaleric acid was prepared from cyclopentanone *via* cyclopentanoneoxime according to the method of Vallach (*5*). DL-Alanine, L-glutamate, L-valine, L-lysine were obtained commercially. *T. utilis* was grown for 20 hours at 30°C in a glucose-salts medium (*1*). It was harvested by centrifugation and, after washing once with water, used for the subsequent reaction.

Incubation of T. utilis with DL-α-AAA-6-C^{14}—A half ml. of cell suspension of *T. utilis* (80 mg. wet weight) was added to 1.2 ml. of the medium containing 5 μmoles of DL-α-AAA-6-C^{14}, 100 μmoles of glucose and 0.2 ml. of *M*/15 potassium phosphate buffer (pH 6.0). The mixture was incubated for 1 hour (experiment 1) or 2 hours (experiment 2) at 30°C in a Ogata's flask (*6*) fitted with rubber bungs and glass tubes for gassing mounted on a rotary shaking table. Incoming and exhaust air was freed of carbon dioxide by passing through a tube of sodalime or by scrubbing through solution of sodium hydroxide.

At the end of incubation aliquots of the mixture were immediately transferred to an ordinally test tube and the cells were collected by centrifugation. The supernatant fluid was decanted and the cells were washed twice with cold water.

Analysis of Radioactive Amino Acid—To the stick cells was then added 1.0 ml. of 10% trichloroacetic acid (TCA) and it was placed in a boiling water bath for 15 minutes. When cooled, the coagulated cells were centrifuged and the supernatant was decanted. The treatment was repeated.

The coagulated cells were treated with ethanol and ethanol-ether (1 : 1) and was hydrolyzed in a sealed tube with 6 *N* HCl for 24 hours at 105°C and most hydrochloric acid was removed by evaporation *in vacuo.*

The supernatants were combined and TCA was extracted four times with ether after addition of 0.05 ml. of 6 *N* HCl. The aqueous solution was lyophilized and was subjected to amino acid analysis as follows.

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For the sample of experiment 1, separation of amino acids was performed in three steps. The hydrolyzates of the TCA insoluble fraction as well as the TCA soluble fraction were dissolved in a small amount of water and the pH was adjusted to about 3.5 with 0.5 *N* NH_4OH. Acidic amino acids were separated from basic and neutral amino acids by passing the solution through a column of Amberlite IR-4B (0.7× 30 cm.) (*7*). After washing out basic and neutral amino acids from the column with water, acidic amino acids were eluted with 1 *N* HCl and the eluate was evaporated to dryness *in vacuo* over H_2SO_4 and solid NaOH.

The washing which contained basic and neutral amino acids was dried *in vacuo*, dissolved in a small amount of acetate buffer (pH 5.5, 0.2 *M*) and then treated with a column of Dowex 50 (15×0.9 cm.) according to the method of Hirs *et al.* (*8*). Fractions from these column containing neutral amino acids and each of basic amino acids were collected and ammonium-aectate was removed *in vacuo*.

Separation of amino acids in acidic and neutral amino acid fraction of experiment 1 as well as the soluble fraction of experiment 2 was performed (in a separate run) on a column of Dowex 50 (100×0.9 cm.) according to the method of Wall (*9*), and amino acids in the eluate were identified after removal of hydrochloric acid by one dimentional paper chromatography on "Toyo" No. 50 filter paper by overnight irrigation with phenol-H_2O (77 : 23 v/v), *n*-butanol-acetic acid-H_2O (4.5 : 1 : 2 v/v) or pyridine-H_2O (65 : 35 v/v.).

Further purification of the isolated lysine was performed by one dimentional paper chromatography with *n*-butanol-acetic acid-H_2O. The paper was dried in air. The position of lysine located was cut out, eluted with water and aliquots of the eluate were subjected to the estimation of lysine and radioactivity.

Degradation of δ-Aminovaleric Acid and Measurement of Radioactivity—Degradation of δ-aminovaleric acid was carried out by the method of Strassman and Weinhouse (*3*). For counting, samples were plated in duplicate on stainless steel disks. Radioactivity was measured with a Geiger-Müller tube with a thin mica window.

RESULTS AND DISCUSSION

Distribution of Radioactivity—To a medium containig α-AAA-6-C^{14} was added washed cells of *T. utilis* and it was incubated at 37°C. The distribution of radioactivity in various fractions as well as in each of amino acid fractions is recorded in Table I. It will be noted that in experiment 1 about 50 per cent of radioactivity of the soluble fraction was found in acidic fraction and about 44 and 6 per cent were present in basic and neutral fractions, respectively. Percentage of radioactivity increased for basic and neutral fraction and decreased for acidic fraction during the course of incubation. These results indicate that most of α-AAA-6-C^{14} metabolized was converted to the form of basic amino acid, along with small amount of neutral amino acid.

TABLE I

Distribution of Radioactivity in Yeast after Incubation with α-AAA-6-C^{14}

Experiment No.			C.P.M.	Fraction	C.P.M.
1[a]	Cells	TCA sol.	7,610	Acidic	3,540
				Neutral	460
				Basic	3,170
		TCA insol.	2,550	Acidic	900
				Neutral	—
				Basic	930
	Medium		24,760		
2[b]	Cells	TCA sol.		Acidic	2,290
				Neutral	1,060
				Basic	6,070
		TCA insol.			3,930
	Medium		12,500		

[a] Incubated for 1 hour.

[b] Incubated for 2 hours.

In these experiments, the respiratory carbon dioxide was recovered over the period of incubation and assayed for radioactivity. The total radioactivity in the carbon dioxide was found to have no significant value.

Separation of Radioactive Amino Acids—The locations of radioactivity and of amino acids obtained in typical runs by means of the column chromatography are shown in Fig. 1. The radioactivity of acidic amino acids in TCA soluble fraction of experiment 1 was attributable to only α-AAA-6-C^{14}, and no significant radioactivity was observed for the positions correspond to glutamate and aspartate, respectively. Three amino acids were formed from DL-α-AAA-6-C^{14} in a course of 1 hour incubation with whole cells of *T. utilis* and the major amino acid formed was lysine.

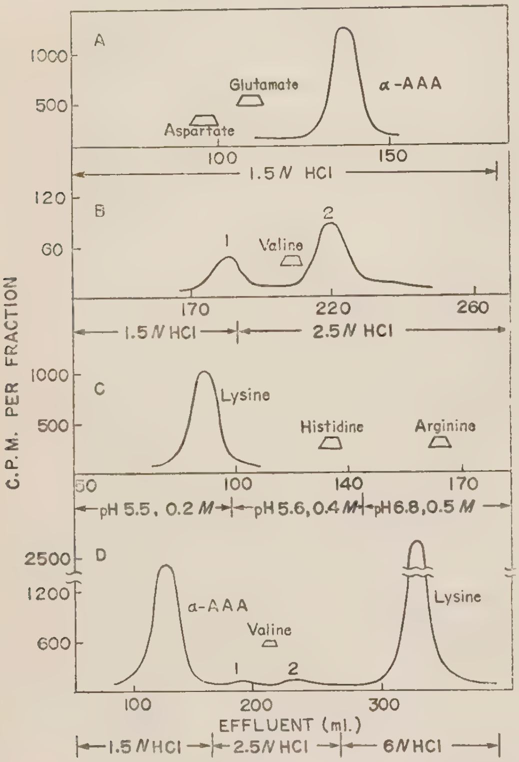


Fig. 1. Separation of radioactive amino acids by ion-exchange chromatography.

A) Acidic fraction of experiment 1: Dowex 50, 105×0.9 cm.
B) Neutral fraction of experiment 1: Dowex 50, 80×0.9 cm.
C) Basic fraction of experiment 1: Dowex 50, 16×0.8 cm.
D) Soluble fraction of experiment 2: Dowex 50, 95×0.9 cm.

Fractions of 4 ml. were collected and the curve was determined by analysis of aliquots of the fraction.

Specific Activity of the Isolated Lysine—Specific activity of the lysine-C^{14} isolated from the TCA soluble fraction of experiment 2 is presented in Table II. The ratio of specific activity of lysine to that of α-AAA was 0.728. From a consideration of dilution effect with endogeneous lysine which both preexisted and was synthesized *de novo* during the incubation, it is clear that the carbon chain of α-AAA-6-C^{14} was metabolized entirely into lysine (lysine-ε-C^{14}). These observations are in accord with the results obtained in our previous paper (*1*).

Table II

Specific Activity of Lysine-C^{14} Formed from α-AAA-6-C^{14}

Amino acid	α-AAA-6-C^{14}	Lysine-C^{14}
C.P.M. per μmole	15,900	11,580
Ratio: Lysine/α-AAA	0.728	

Identification of δ-Aminovaleric Acid—As shown in Fig. 1 B, two distinct peaks were observed on a ion-exchange chromatogram of neutral amino acid fraction obtained from the incubation mixture with DL-α-AAA-6-C^{14}. One (peak 1) appears before valine and other (peak 2) after. When the activity was tested individually, both accounted for essentially all of the activity found in the neutral amino acid fraction. With the use of ion-exchange chromatography, it has been demonstrated that the two amino acids are different from known common amino acids. As the compound appeared in peak 2 seemed to be δ-aminovaleric acid from the behavior on paper (Table III) and ion-exchange chromatography, it was recrystallized five times with authentic specimen from water-ethanol. These results as well as isolated lysine of experiment 2 are shown in Table IV. The activity was constant after the fifth recrystallization and therefore, the compound was identified as δ-aminovaleric acid. The compound appeared in peak 1 on the other hand, was presumed to be α-hydroxy-ε-aminohexanoic acid from the chromatographic behaviour, but we could obtained the compound in too small quantities to make a definite conclusion.

Two metabolic pathways are possible to explain the formation of δ-aminovaleric acid from α-AAA, *i.e.*, one is decarboxylation of α-AAA with loss of α-carboxyl carbon, and

TABLE III

R_f Values of Unknown Amino Acids Formed from α-AAA-6-C^{14}

Solvent v/v	R_f		
	BuOH-AcOH-H_2O 4.5 : 1 : 2	Phenol-H_2O 77 : 23	Pyridine-H_2O 65 : 35
Lysine	0.12—0.14	0.43	
δ-Aminovaleric acid	0.38—0.40	0.80	0.52—0.57
α-Hydroxy-ε-aminohexanoic acid	0.28—0.32	0.60—0.79	0.54—0.56
Piperidine-2-carboxylic acid	0.43—0.44	0.95	0.67—0.79
Unknown, in peak 1[a)]	0.28—0.33	0.63—0.74	0.56
Unknown, in peak 2[a)]	0.37	0.75—0.83	0.52—0.57

a) Position of radioactivity located.

TABLE IV

Carrier Recrystallization of Radioactive Lysine and δ-Aminovaleric Acid

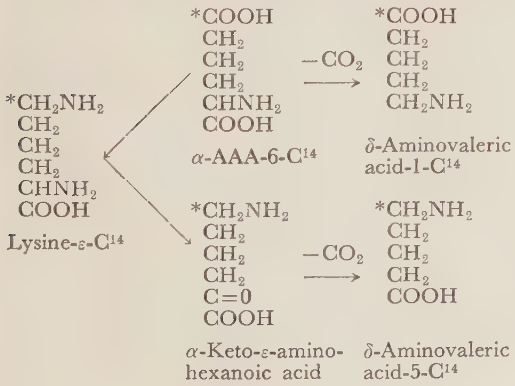
Crystallization[1)]	Lysine		δ-Aminovaleric acid	
	Yield (mg.)	C.P.M. per. mg.	Yield (mg.)	C.P.M. per 10 mg.
First	43.0	78.3	37.4	22.4
Second	34.5	86.1	31.0	23.7
Fourth	20.7	78.9	25.8	21.6
Fifth	12.6	77.5	14.5	22.3

1) Recrystallized from water-ethanol.

other is degradation of lysine, presumably via its keto analogue with loss of α-carboxyl carbon of α-AAA. In the former case carboxyl carbon of δ-aminovaleric acid formed will be radioactive, and in the latter case the carboxyl carbon will be nonlabeled as shown in Scheme I.

SCHEME I

Possible Mechanism of δ-Aminovaleric Acid Formation



To elucidate these situations, carboxyl carbon of the δ-aminovaleric acid was converted to $BaCO_3$, but no radioactivity was found in the carbonate. This result excludes the first assumption mentioned above and, therefore, it may be concluded that the fate of α-AAA-6-C^{14} must have been the formation of lysine-ε-C^{14} and then the lysine-ε-C^{14} formed contributed to the formation of both δ-aminovaleric acid and α-hydroxy-ε-aminohexanoic acid via its keto form (Scheme I). The formation of δ-aminovaleric acid from lysine has been reported with a soil bacterium by Kamahora *et al.* (*10*).

Metabolism of D-α-AAA in T. utilis—Additional trials concerned with the utilization of D-α-AAA-6-C^{14} with *T. utilis* were carried out, in which α-AAA-6-C^{14} used was a substance which remained in the reaction mixture of the experiment 2 by further incubation with fresh *T. utilis* cells to remove L-α-AAA-6-C^{14}. The remaining fraction contained almost a half of total activity of the initial DL-α-AAA-6-C^{14}. As illustrated in Table V, the extent in which D-α-AAA-6-C^{14} enters into reaction in *T. utilis* has no significance, as compared to the data with DL-α-AAA-6-C^{14} (Table I). This fact indicates a preferential utilization of L-α-AAA-6-C^{14} in *T. utilis* for lysine biosynthesis, in contrast to the availability of D-α-AAA in lysineless mutant of *N. crassa* (*11*).

From the all results presented here, the metabolic fate of α-AAA in *T. utilis* will be summarized as in Scheme II.

Table V

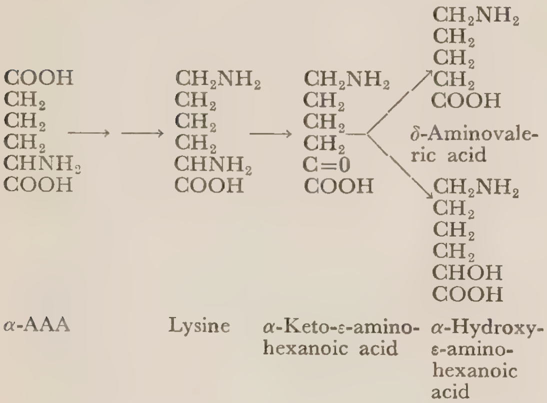
Metabolism of D-α-AAA-6-C^{14} in T. utilis

Incubation time (hour)	Radioactivity (C.P.M.)		
	Cells[1)]		Medium
	Soluble	Insoluble[2)]	
36	99	22	9,000
2	22	16	9,200
2	16	20	9,200

1) Values per 10 mg. wet weight cells.
2) Estimated after hydrolysis.

Scheme II

Metabolic Fate of α-AAA in T. utilis


COOH CH2 CH2 CH2 CHNH2 COOH
α-AAA
CH2NH2 CH2 CH2 CH2 CHNH2 COOH
Lysine
CH2NH2 CH2 CH2 CH2 C=0 COOH
α-Keto-ε-amino-hexanoic acid
CH2NH2 CH2 CH2 CH2 COOH
δ-Aminovaleric acid
CH2NH2 CH2 CH2 CH2 CHOH COOH
α-Hydroxy-ε-amino-hexanoic acid

SUMMARY

1. Metabolic fate of DL-α-AAA-6-C^{14} in *T. utilis* was investigated in connection with lysine biosynthesis.

2. A large amount of lysine was formed from DL-α-AAA-6-C^{14} and the both amino acids had nearly the same specific activity, indicating a direct pathway of α-AAA to lysine.

3. A small amount of radioactivity was also found in δ-aminovaleric acid and possibly α-hydroxy-ε-aminohexanoic acid. The two compounds were suggested to be catabolic products of lysine formed from α-AAA-6-C^{14}.

4. Only L-form of α-AAA was utilized for lysine biosynthesis in *T. utilis*.

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Glutamic Acid Formation from Glucose by Bacteria

V. Oxidative Metabolism of Glucose in *Brevibacterium flavum* No. 2247*

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On the oxidative metabolism of glucose in *Brevibacterium flavum* No. 2247, following results have been obtained (*1–4*): (a) Resting cells oxidized glucose, yielding αKG** and CO_2, and the reaction proceeded stoichiometrically. (b) In the presence of ammonium salts, L-glutamate was formed instead of αKG, from glucose aerobically. If $C^{14}O_2$ was added to this system, C^{14} was fixed into the α-carboxyl carbon of glutamate formed. (c) The relative specific radioactivities and the C^{14}-distribution of pyruvate formed from glucose-1-, -6-, and -U-C^{14} indicated that glucose was metabolized aerobically by both the Embden-Meyerhof- and the hexosemonophosphate oxidative pathway in the ratio of 9 : 1. (d) All enzymes of the TCA cycle, of the glyoxylate bypass, and of glycolysis, except for α-ketoglutaric dehydrogenase and enzymes related to C_3-compounds, were found to be present in the cell-free extracts. These results suggest that in this organism glucose is oxidized to αKG *via* the glycolytic pathway and subsequently *via* the TCA cycle with an additional glyoxylate bypass (*5*).

The present study deals with the inhibitory action of some chemicals on the glucose metabolism as well as with the presence in the cell-free extracts of enzymes related to C_3-compounds.

METHODS

Chemicals—DL-Glyceraldehyde-1-bromide-3-phosphoric acid dioxane complex, cyclohexylammonium dihydroxyacetone phosphate-dimethylketal monohydrate, barium D-3-phosphoglycerate, tricyclohexylammonium phosphopyruvate, CoA, DPN, TPN, GSH, ADP, pyruvic acid, and αKG were purchased from the California Corporation for Biochemical Research. Dihydroxyacetone phosphate was obtained by the hydrolysis of its dimethylketal in 0.1 *N* HCl at 80°C for 10 minutes.

Chemical Determination—αKG and pyruvate were colorimetrically determined as their DiNPH by the slightly modified method of Friedemann and Haugen (*6*) at 420 and 540 mμ, or by the method described previously (*7*), including a paperchromatographic separation of keto acids. Method for the determination of citrate was essentially the same as that of Natelson *et al.* (*8*, *9*). Succinate was determinated manometrically by the specific succinoxidase preparation (*10*).

Enzyme Assay—The methods for the demonstration of GAP dehydrogenase, triose phosphate isomerase, PGA kinase, PGA mutase, enolase, pyruvic oxidase, and pyruvic kinase, were essentially the same as those described in "Methods in Enzymology" Vol. I (*11*).

Oxygen uptake as well as carbon dioxide evolution was measured manometrically. The methods for the preparation of washed cell suspension and of cell-free extracts, determination of the nitrogen content of cell materials, and determination of glucose were described previously (*1*, *2*, *4*).

RESULTS

Oxidation of Glucose in the Presence of Inhibition by Resting Cells—As described previously, glucose was rapidly oxidized by the resting cells and αKG was accumulated as an end product. The net reaction could be represented as follows:

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* The study was partially reported at 32nd General Meeting of the Japanese Biochemical Society (1959).

** The abbreviations used in this paper include αKG, α-ketoglutarate; DiNPH, 2, 4-dinitrophenylhydrazine or 2, 4-dinitrophenylhydrazone; FAc, monofluoroacetate; GAP, glyceraldehyde-3-phosphate; DPN, diphosphopyridine nucleotide; GSH, glutathione; ADP, adenosine diphosphate; PGA, D-3-phosphoglycerate; TPN, triphosphopyridine nucleotide; CoA, coenzyme A; TPP, thiamine pyrophosphate.

$$C_6H_{12}O_6+4O_2=3.5CO_2+0.5C_5H_6O_5+4.5H_2O$$

When some kinds of chemicals were added to this reaction system, the rate of oxygen uptake as well as that of glucose consumption decreased more or less and, in some cases, oxygen absorption was stopped at a lower level than that expected from the above equation, as shown in Table I.

Since the latter results seemed to suggest the accumulation of a certain intermediate of the reaction producing αKG from glucose, the reaction mixtures were examined by paper-chromatography. Thus, pyruvate and citrate were detected in the cases where sodium arse-

TABLE I

Effect of Inhibitors on the Glucose Metabolism

Inhibitor	Concentration $M\times10^3$	Inhibition of		Total O_2 uptake 3) mole/mole glucose
		Q_{O_2} 1) %	glucose consumption 2) %	
p-Chloromercuribenzoate	1	100	100	—
CH_2ICOOH	1	100	100	—
$NaAsO_2$	10	56	21	—
〃	50	—	—	1
KCN	1	82	−36	—
8-Hydroxyquinoline	1	14	5	—
Thiourea	1	15	−7	—
α, α'-Dipyridyl	1	15	−4	—
o-Phenanthroline	1	53	69	—
NaF	1	6	5	—
〃	25	—	—	2
EDTA	1	−5	11	2
Nitroso-R-salt	1	5	−7	—
o-Phenylenediamine	1	0	−6	2
NaN_3	1	7	7	—
Semicarbazide	1	3	5	2
Hydroxylamine	1	35	7	—
〃	10	—	—	2
2, 4-Dinitrophenol	1	−6	−2	—
Arsenate	10	31	9	1.5
Diethyldithiocarbamate	1	2	9	—
Malonic acid	50	46	4	1
CH_2FCOOH	10	27	60	—
〃	50	—	—	2.5
Dihydrostreptomycine	20μg./ml.	−14	−4	—
Penicilline G	10unit/ml.	0	−12	—

1) Reaction mixture contained 30 μmoles of glucose, 100 μmoles of phosphate buffer, pH 7.0 (or pH 5.8 in the case of malonic acid), 0.2 mg. N of resting cells, inhibitor described above, and water in a final volume of 1 ml. Total volume of reaction mixture per Warburg vessel was 2.5 ml. Shaken at 37°C.

2) Composition of reaction mixture was the same as that of 1). Total volume of reaction mixture per Monod tube was 5 ml. Consumed glucose was estimated after 3 hours' aerobic incubation at 37°C.

3) Conditions were the same as that of 1) except for glucose content; 2 μmoles per vessel, instead.

nite and sodium monofluoroacetate were added, respectively.

Pyruvate was identified as main product of the arsenite-inhibited reaction, by chromatographic behaviour, characteristic absorption spectrum (Fig. 1), and melting point of its DiNPH.

	Melting point (uncorrected)
Authentic pyruvate-DiNPH	208.3°C
Product-DiNPH	207.9°C
Mixture of above two specimens	208.5°C

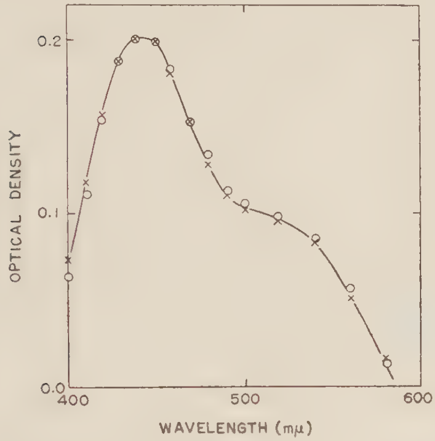

FIG. 1. Absorption spectra of 2, 4-dinitrophenylhydrazones of pyruvate (—O—) and carbonyl compound accumulated in the presence of sodium arsenite when glucose was oxidized by the resting cells (—×—).
Solvent: 1 *N* NaOH

While the paperchromatography indicated the product of the FAc-inhibited reaction to be citrate, this result was further confirmed after the conversion of the product to pentabromoacetone by the same method as that used for the quantitative determination of citrate. Yellowish white precipitates, which seemed to be pentabromoacetone, were collected by filtration and recrystallized twice by dropwise addition of water to their ethanol solution. The melting point of the crystalline product thus obtained as well as the absorption spectrum of its thiourea-complex (Fig. 2) was identical with that of pentabromoacetone prepared from authentic citrate by the same procedures, namely:

	Melting point (uncorrected)
Authentic pentabromoacetone	72.5°C
Product isolated from reaction mixture	72.7°C
Mixture of above two specimens	72.5°C

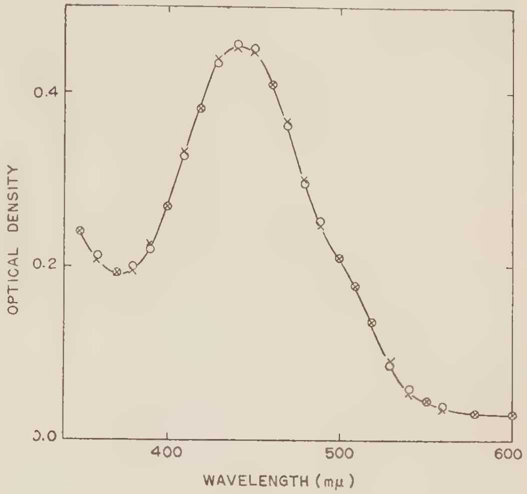

FIG. 2. Absorption spectra of thiourea-pentabromoacetone complexes derived from citrate (—O—) and organic acid accumulated in the presence of CH_2FCOOH when glucose was oxidized by the resting cells (—×—).
Solvent: 1 *N* NaOH

Effect of Inhibitors on the Accumulation of Some Metabolites—After glucose was oxidized by the resting cells in the presence of arsenite and/or FAc, the product mentioned above as well as αKG in the reaction mixters were determined as shown Table II a.

TABLE II a

Inhibitors and Products in the Oxidation of Glucose

Inhibitor		Concentration of substrate and product after 5 hrs. (μmole per ml.)			
$NaAsO_2$	$CH_2FCOONa$	Glucose	Pyruvate	Citrate	αKG
−	−	−0.1	−0.3	0.2	10.3
−	+	1.4	1.7	3.0	0.5
+	−	0.4	10.4	0.0	0.4
+	+	0.0	10.9	0.1	0.1

Reaction mixture contained 20 μmoles of glucose, 100 μmoles of phosphate buffer, pH 7.0, 50 μmoles of inhibitor, 0.42 mg. N of resting cells, and water in a final volume of 1 ml. Incubated aerobically at 37°C for 5 hours.

Thus, it was clearly demonstrated that the formation of αKG was strongly inhibited by adding arsenite and/or FAc, and that the main products from glucose were pyruvate and citrate in the presence of arsenite and FAc, respectively. It was of interest that, in the system containing both arsenite and FAc, the main product was pyruvate and the amount of the product was almost the same as that observed in the system containing arsenite alone.

TABLE II b

Inhibitors and Products in the Oxidation of Pyruvate

Inhibitor		Concentration of substrate and product after 5 hrs. (μmole per ml.)		
$NaAsO_2$	$CH_2FCOONa$	Pyruvate	Citrate	αKG
–	–	7.1	2.0	5.3
–	+	24.0	4.6	0.1
+	–	34.7	0.1	0.2

Reaction mixture contained 40 μmoles of sodium pyruvate, 100 μmoles of phosphate buffer, pH 7.0, 50 μmoles of inhibitor, 0.27 mg. N of resting cells, and water in a final volume of 1 ml. Incubated aerobically at 37°C for 5 hours.

Pyruvate, one of the intermediates of glucose metabolism, was also rapidly oxidized by the resting cells, to form αKG. The effect of FAc on the pyruvate metabolism was similar to that on the glucose metabolism described above. A strong inhibition of the pyruvate metabolism was caused by arsenite, where neither αKG nor citrate was formed (Table II b).

Formation of Succinate under Semi-aerobic Condition—Under certain semi-aerobic condition, which was established by increasing the volume of the reaction mixture relative to the air space of tube, the resting cells metabolized glucose to form succinate (Table III).

The less aeration resulted in the less accumulation of αKG and in the more accumulation of succinate. Succinate was identified by the paperchromatographic behaviors (solvent systems: *n*-butanol-formic acid-water (4 : 1.5 : 1), isoamylalcohol-4 *M* formic acid (1 : 1), and ethanol-conc. ammonia water-water (80 : 5 : 15)) and by the oxygen uptake with succinoxidase preparation. Formation of succinate from glucose was also strongly inhibited by arsenite with concomitant accumulation of pyruvate in the reaction mixture (Table IV).

TABLE III

Effect of Aeration on Glucose Metabolism

Volume of reaction mixture per Monod tube	Concentration of product after 5 hrs. (μmole per ml.)		
	Succinate	αKG	Pyruvate
5 ml.	0.3	8.9	0.4
20 〃	2.5	2.5	2.1
30 〃	3.9	1.6	0.6

Reaction mixture contained 20 μmoles of glucose and 0.39 mg. N of resting cells in a final volume of 1 ml. of 0.1 *M* phosphate buffer, pH 7.0. Shaken in a Monod tube at 37°C for 5 hours.

TABLE IV

Effect of Arsenite on Succinate Formation from Glucose

Arsenite $M \times 10^{-2}$	Concentration of substrate and product after 5 hrs. (μmole per ml.)		
	Glucose	Pyruvate	Succinate
0	0.1	2.2	4.4
5	8.2	6.0	0.8

Reaction mixture contained 20 μmoles of glucose, 0.32 mg. N of resting cells, and arsenite in a final volume of 1 ml. of 0.1 *M* phosphate buffer, pH 7.0. Total volume of reaction mixture was 30 ml. per Monod tube (T-type, diameter: 16 mm. length: 250 mm.).

Demonstration of the Enzymes Related to the Metabolism of Three-carbon Compounds in the Cell-free Extracts—DPN reduction occurred upon incubation of GAP with the sonic cell extracts in the presence of arsenate and of GSH, indicating the presence of GAP dehydrogenase. When dihydroxyacetone phosphate was incubated with the cell extracts under the same conditions as described above, reduction of

DPN could be demonstrated spectrophotometrically. The result seems to indicate the presence of triose-phosphate isomerase, since arsenate and GSH were required for the DPN reduction by dihydroxyacetone phosphate, as by GAP. DPN reduction occurred also in the presence of phosphate and ADP instead of arsenate with the testing system of GAP dehydrogenase. The presence of ADP was essential for the reaction, indicating the presence of PGA kinase. Spectrophotometric studies of the hydration and the formation from PGA of phosphopyruvate demonstrated the existence of enolase as well as PGA mutase. Magnesium ions were required for full activity. Studies of the reduction of pyridine nucleotides with pyruvate in the presence of dried cell extracts indicated the presence of DPN-specific pyruvic oxidase, which required CoA, TPP, and magnesium ions for maximum activity. The presence of pyruvic kinase was demonstrated by the fact that phosphopyruvate could be used in place of pyruvate for DPN reduction under the same condition as described above, when ADP was added (Table V).

DISCUSSION

Under certain condition, where the resting cells of *B. flavum* oxidized glucose to form αKG and CO_2, the addition of arsenite or of monofluoroacetate caused the accumulation of pyruvate or citrate, respectively, instead of αKG. In the presence of inhibitors, oxygen uptake was stopped at a lower level than that observed in their absence. Therefore, it may be concluded that pyruvate as well as citrate is an intermediate in the αKG formation reaction from glucose. Moreover, when the resting cells oxidized glucose in the presence of FAc to form citrate, further addition of arsenite caused the accumulation of pyruvate instead of citrate. These results suggest the following metabolic sequence of glucose:

Glucose→Pyruvate→Citrate→α-Ketoglutarate

Formation of pyruvate has been also observed at the initial stage of aerobic metabolism of glucose (*2*).

It was previously concluded from isotopic experiments that the main path of pyruvate formation from glucose is the Embden-Meyerhof pathway (*3*). This conclusion was strongly supported by both the present and previous results (*1*) which demonstrated the presence of all of the enzymes of the Embden-Meyerhof pathway in the cell-free extracts used. The diminished rate of oxidation and the accumulation of pyruvate when glucose is metabolized in the presence of arsenite suggests that pyruvate oxidation is mediated by a lipoic acid complex (*12*, *13*). Thus, the experiments with cell-free extracts demonstrated DPN-specific pyruvic oxidase requiring CoA, $MgCl_2$, and TPP for full activity, similarly as observed with pyruvic oxidase of *E. coli*, which contained also lipoic acid as coenzyme (*14*, *15*).

FAc is known to be activated and to condense to form fluorocitrate, which inhibits aconitase activity and so causes the accumulation of citrate (*16–18*). Although citrate was accumulated when glucose oxidation was inhibited by FAc, it could not be oxidized by the cell suspension even in the absence of FAc. However, the position of citrate in the metabolic pathway of glucose was further clarified by the detection of powerful aconitase and isocitric dehydrogenase activity in the cell extracts. All other enzymes of the TCA cycle with the exception of α-ketoglutaric dehydrogenase have been demonstrated in this organism (*1*, *5*). From these results, there seems little doubt that terminal respiration in this organism occurs *via* the modified TCA cycle with an additional glyoxylate bypass as described previously (*4*, *5*).

SUMMARY

1. When the resting cells of *Brevibacterium flavum* No. 2247 oxidized glucose to form α-ketoglutarate and CO_2, addition of arsenite or of monofluoroacetate caused the accumulation of pyruvate or of citrate, respectively, instead of α-ketoglutarate. The simultaneous presence of both inhibitors caused also the accumulation of pyruvate. From thes results, following metabolic sequence of glucose was

Table V

Demonstration of the Enzymes Related to the Metabolism of Three-Carbon Compounds in Cell-Free Extracts

Enzyme related	Substrate	Reaction mixture and condition	Reaction rate
GAP dehydrogenase	DL-Glyceraldehyde-3-phosphate		ΔE_{340}/min.
		Complete sys.-I, 26°C	1.0×10^{-2}
		—substrate 〃	0.0 〃
		—arsenate,, 〃	0.0 〃
		—glutathione, 〃	0.05 〃
Triose-phosphate isomerase and GAP dehydrogenase	Dihydroxyacetone-phosphate		ΔE_{340}/min.
		Complete sys.-II, 26°C	0.90×10^{-2}
		—substrate, 〃	0.00 〃
		—arsenate, 〃	0.05 〃
		—glutathione, 〃	0.00 〃
		—enzyme, 〃	0.00 〃
PGA kinase and GAP dehydrogenase	DL-Glyceraldehyde-3-phosphate		ΔE_{340}/min.
		Complete sys.-III, 26°C	8.5×10^{-2}
		—substrate, 〃	0.0 〃
		—ADP, 〃	0.0 〃
Enolase	Phosphopyruvate		$(-\Delta E_{240})$/min.
		Complete sys.-IV, 25°C	1.2×10^{-2}
		—substrate, 〃	0.0 〃
		—enzyme, 〃	0.0 〃
PGA mutase and enolase	D-3-Phosphoglycerate		ΔE_{240}/min.
		Complete sys.-V, 27°C	0.88×10^{-2}
		—substrate, 〃	0.00 〃
		—$MgCl_2$, 〃	0.22 〃
		—enzyme, 〃	0.00 〃
Pyruvic oxidase	Pyruvate		ΔE_{340}/min.
		Complete sys.-VI, 25°C	1.30×10^{-2}
		—substrate, 〃	0.00 〃
		—CoA, 〃	0.35 〃
		—$MgCl_2$, 〃	0.35 〃
		—TPP, 〃	0.00 〃
		—DPN+TPN, 〃	0.06 〃
Pyruvic kinase and pyruvic oxidase	Phosphopyruvate		ΔE_{340}/min.
		Complete sys.-VII, 25°C	1.9×10^{-3}
		—substrate, 〃	0.1 〃
		—ADP, 〃	0.2 〃
		—KCl, 〃	6.0 〃

Complete systems contained the following reactants in micromoles per ml.

Complete system-I: GAP 0.5; Tris. buffer, pH 7.5, 50; DPN 0.125; Na-arsenate 10; glycine 25; GSH 7.5; and sonic cell extract (dialysed for 2 hrs.) 0.158 mg. N.

Complete system-II: dihydroxyacetone phosphate 0.25; Tris. buffer, pH 7.5, 50; DPN 0.125; Na-arsenate 10; glycine 25; GSH 7.5; and sonic cell extract (dialysed for 2 hrs.) 0.158 mg. N.

Complete system-III: GAP 0.5; K-phosphate buffer, pH 7.0, 50; DPN 0.125; ADP 1; $MgCl_2$ 5; GSH 7.5; glycine 25; and dried cell extract (dialysed for 4 hrs.) 0.021 mg. N.

Complete system-IV; phosphopyruvate 0.2; Tris. buffer, pH 7.5, 50; $MgCl_2$ 2.5; glycine 25; and sonic cell extract (dialysed for 2 hrs.) 0.011 mg. N.

Complete system-V: PGA 5; Tris. buffer, pH 7.5, 50; $MgCl_2$ 2.5; glycine 25; and sonic cell extract (dialysed for 2 hrs.) 0.0043 mg. N.

Complete system-VI: K-pyruvate 2.5; Tris. buffer, pH 7.5, 50; DPN 0.125; CoA 0.081; TPP 0.15; $MgCl_2$ 5; GSH 5; and dried cell extract (dialysed for 2 hrs.) 0.102 mg. N.

Complete system-VII: phosphopyruvate 2.5; Tris. buffer, pH 7.5, 50; DPN 0.125; ADP 1; CoA 0.081; TPP 0.15; $MgCl_2$ 5; KCl 75; GSH 5; and dried cell extract (dialysed for 2 hrs.) 0.102 mg. N.

suggested: glucose / pyruvate / citrate / α-ketoglutarate. Under certain semiaerobic condition, succinate was accumulated instead of α-ketoglutarate.

2. An evidence was obtained for the presence of DPN-linked glyceraldehyde-3-phosphate dehydrogenase, triose-phosphate isomerase, 3-phosphoglyceric kinase, phosphoglycerate mutase, enolase, pyruvic kinase, DPN-linked pyruvic oxidase in the cell-free extracts of this organism.

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Enzymic Studies on Mannitol Formation by *Piricularia oryzae**, **

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Mannitol is one of the most widely occurring hexahydric polyol in nature. This hexitol is not only accumulated by many microorganisms as a reserve material or as a fermentation product, but also found in many plants, especially in algae. Although it has been supposed that mannitol is synthesized in these organisms from sugar by enzymic reduction, precise mechanism of formation of this hexitol has remained unclarified. *Piricularia oryzae* CAVARA, a fungus causing the blast disease of the rice plant, was found to accumulate a considerable amount of mannitol when it was grown in the culture media containing several kinds of sugars. In the present paper this fungus was used to study the mechanism of formation of mannitol. The results obtained here proved that in *P. oryzae* mannitol was produced from F-6-P**** in the following way: F-6-P was first reduced enzymically to M-1-P***** in the presence of DPNH, then resulting M-1-P was hydrolyzed to mannitol by a specific phosphatase.

EXPERIMENTALS

Substrates—M-1-P was prepared by the direct phosphorylation of mannitol (*2*). This M-1-P preparation was contaminated with an unknown phosphate ester which was detectable by paper chromatography and only 70 per cent were proved to be available as a substrate for the following F-6-P reducing enzyme. M-1-P was also prepared by chemical reduction of mannose-6-phosphate (*3*). Sorbitol-6-phosphate and dulcitol-6-phosphate were prepared by the chemical reduction of G-6-P and galactose-6-phosphate respectively (*3*). The sorbitol-6-phosphate preparation was contaminated with 8 per cent of M-1-P and all the experimental data obtained by using this sorbitol-6-phosphate preparation were corrected for this contamination. Fructose-1,6-diphosphate and F-6-P were prepared according to Neuberg (*4*). Glucose-1-phosphate was cordially given by Mr. Hamada of Mie Prefectural University. Other materials used were commercial products. All the barium and calcium salts were converted to free acids by the treatment with H-form of Amberlite IR-120.

Coenzymes—DPN (purity 70 per cent) was prepared by the method of Okunuki *et al.* (*5*). TPN (purity 80 per cent) was prepared by LePage's method modified by Yamada *et al.* (*6*). DPNH (purity 65 per cent) was prepared by the enzymic reduction of DPN with alcohol dehydrogenase system followed by precipitation as its sodium salt.

Analytical Methods—All the spectrophotometric measurements were made by Beckman spectrophotometer model DU. Hexitol and hexitol phosphate were assayed by the method of Burton (*7*). F-6-P was assayed by Roe's method (*8*). Reducing sugar was determined by Nelson's method (*9*). Inorganic phosphate was determined by Barton's method (*10*). Protein was estimated by UV method (*11*) or Biuret method (*12*).

Microorganisms—*P. oryzae* (No. 9-5828) and *Aspergillus niger* used were stock strains in this laboratory. *Aspergillus oryzae* was kindly furnished by Mr. Kami-

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* A preliminary note on this work has been reported previously (*1*).

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**** Abbreviations: F-6-P, fructose-6-phosphate; G-6-P, glucose-6-phosphate; M-1-P, mannitol-1-phosphate; M-1-Pase; mannitol-1-phosphatase; DPN, DPNH, oxidized and reduced diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; EDTA, ethylenediamine tetraacetate; PCMB, *p*-chloromercuribenzoate.

***** Owing to its symmetrical structure, mannitol phosphate produced from F-6-P should be named M-1-P.

hara, *Neurospora crassa* and *Neurospora sitophila* were also furnished by Dr. Imai of Kyoto University.

Growth of Fungi—*P. oryzae* and other fungi used throughout this experiment were grown at 28°C without shaking in a synthetic medium containing sucrose, mineral salts, thiamine and biotin (*13*). The mycelia were harvested by filtration, washed and frozen until use.

Preparation of Cell-Free Extracts—In a chilled mortar frozen mycelia were ground with twice the weight of powdered alumina (Wako No. 800) for 5 minutes and resulting paste was extracted with three times the original weight of cold water. After removal of alumina by light centrifugation (at 3,000×*g*, 5 minutes) the turbid extracts were centrifuged at 10,000×*g*, for 15 minutes. The supernatant fluid was referred to the cell-free extracts in this paper. In the case of the determination of hexitol and sugar in mycelia, the paste was extracted with the same amount of 0.1 *M* Na_2CO_3 instead of cold water.

RESULTS

Accumulation of Mannitol by the Fungus

P. oryzae was grown in a medium containing sucrose as the sole carbon source. At intervals samples of the mycelia and of the culture filtrate were taken separately, then cell-free extracts were prepared from the samples of mycelia. The culture filtrates and the cell-free extracts were hydrolyzed in 0.05 *N* HCl at 74°C for 70 minutes and subjected to assay for hexitol and reducing sugar. The amount of hexitol accumulated in mycelia was found to be about 10 per cent of dry weight of the mycelia and the content showed no appreciable change throughout the growth stages of the fungus (Fig. 1). The amount of reducing sugar in mycelia was only trace. In the filtrate no hexitol was detected. The hexitol accumulated in the mycelia of *P. oryzae* has previously been identified as mannitol by Takagi* in this laboratory.

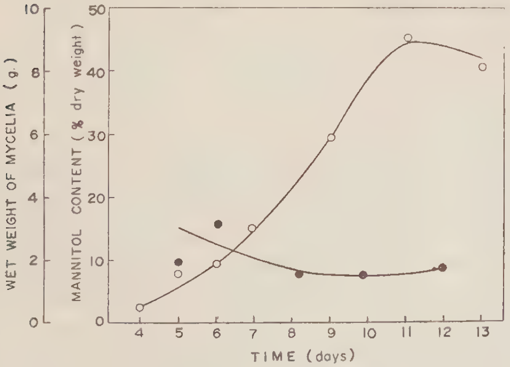

Fig. 1. The content of mannitol in mycelia at different growth stage of *P. oryzae*.

—○— Growth, —●— Mannitol content.

Enzymic F-6-P Reduction Followed by Dephosphorylation

In the course of the studies of G-6-P dehydrogenase of *P. oryzae* using crude extracts of mycelia, the rapid oxidation of DPNH depending on the presence of G-6-P was observed. F-6-P was found to be superior substrate to G-6-P for this enzymic reduction. Free fructose or glucose was not reduced unless adenosine triphosphate was added to the system. In order to isolate the reaction product of the enzymic reduction of F-6-P, large scale experiment was carried out. Toward the end of incubation larger quantity of inorganic phosphate was found to be formed and only a small amount of organic phosphate compound remained. Coexistence of active phosphatase was suspected in the enzyme preparation. Since no liberation of inorganic phosphate was observed in the case of incubation of F-6-P without DPNH with the same enzyme preparation, liberation of inorganic phosphate in the above experiment should be attributed to hydrolysis of the intermediary organic phosphate compound formed by the reduction of F-6-P.

F-6-P Reductase

Assay—In 3.0 ml. cuvette 0.2 μmoles of DPNH, 280 μmoles of $NaHCO_3$ and enzyme solution were placed in a final volume of 2.9 ml. Ten μmoles of F-6-P in 0.1 ml. was mixed and the reaction was initiated. The decrease of absorbance at 340 mμ were read at 15 second intervals at 32°C. Standard assay was carried out by the following procedure. In 3.0 ml. cuvette 1 μmole of DPN, 140 μmoles of Tris buffer, pH 10.0 and a suitable amount of enzyme were placed in a total volume of 2.9 ml. The reaction was started by the addition of 10 μmoles of M-1-P in 0.1 ml. and the absorbance at 340 mμ was read at 15 second intervals. The reaction was carried out at 32°C and proceeded linearly for at least few minutes. One unit was defined as the amount of enzyme causing an increase in absorbance of 0.001 from 15 seconds to 75 seconds under the assay conditions. The increase in absorbance was proportional to enzyme concentration within the range up to 300 units of enzyme under the standard assay condition. Specific activity was expressed as units per mg. of protein.

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* Unpublished.

Purification—All the purification procedures were carried out at 0 to 5°C. To 80 ml. of crude extract 2.4 ml. of 1 M $MnCl_2$ was added with stirring and the precipitate was centrifuged off at 10,000 $\times g$ for 15 minutes (Mn fraction). To the clear supernate 1 N KOH was added dropwise until the pH of the solution reached to 9.0 (to thymol blue) and the resulting turbid solution was kept at 50°C for 4 minutes. After cooling in ice bath the voluminous precipitate was centrifuged off at 10,000 $\times g$, for 15 minutes (heated fraction). Ten ml. of the heated fraction was chilled to 0°C and 4 ml. of acetone (−10°C) was cautiously added with stirring. During acetone fractionation the enzyme solution was chilled in an ice-salt mixture and the temperature of the solution was kept below 0°C. After centrifugation at 5,000 $\times g$ for 5 minutes the precipitate was discarded. To the supernate (13 ml.) 6 ml. of acetone (−10°C) was added and centrifuged as above. The supernate was discarded and the precipitate was dissolved in 3.3 ml. of 0.1 M sodium acetate (acetone fraction). The purification thus attained was about 25-fold (Table I).

TABLE I

Purification of F-6-P Reductase

Step	Total volume (ml.)	Units (10^3)	Total protein (mg.)	Specific activity (10^2)
Crude extract	80	133	800	1.66
Mn fraction	80	133	400	3.32
Heated fraction[1]	80	153	48	32.0
Acetone fraction[1]	27	83	20	40.8

Assay conditions are described in the text.

[1] All the values were corrected for the amount used for pilot test during purification steps.

Reaction Product and Stoichiometry—Eleven μmoles of F-6-P and 23 μmoles of DPNH were dissolved in a small quantity of water, the pH of the solution was adjusted to 8.0 by the cautious addition of dilute alkali and the mixture was made up to a total volume of 40 ml. One ml. of F-6-P reductase solution was added and the reaction was initiated. The incubation was carried out at 28°C for 30 minutes, and the reaction was stopped by heating the tube in boiling water bath for 5 minutes. In another tube, as a zero time control, the enzyme was added to a previously heated reaction mixture and the tube was kept in the boiling water for further 5 minutes. The resulting precipitate of the denatured protein was centrifuged off and the clear supernate was subjected to assay for DPNH, F-6-P and hexitol phosphate respectively. The disappearance of F-6-P and DPNH were well balanced with the appearance of hexitol phosphate (Table II). M-1-P and sorbitol-6-phosphate were equally considered as products in this reaction. To determine the structure of the reaction product, M-1-Pase preparation was used as an analytical tool (M-1-Pase is described later in detail). Deproteinized aliquot from the reductase reaction mixture was incubated with 10 μmoles of $MgCl_2$ and 0.1 ml. of M-1-Pase preparation in a total volume of 2.0 ml. The incubation was carried

TABLE II

Stoichiometric Studies of F-6-P Reductase

−ΔDPNH (μmoles)	−ΔF-6-P (μmoles)	ΔHexitol phosphate (μmoles)	Inorganic phosphate liberated by M-1-Pase (μmoles)
7.3	7.8	7.2	7.0

Experimental conditions are described in the text.

out at pH 8.0, at 28°C for 30 minutes and the amount of inorganic phosphate liberated was determined just as in the M-1-Pase assay. The amount of liberated inorganic phosphate was nearly equimolar to that of hexitol phosphate, which had been formed by the enzymic reduction of F-6-P (Table II). Synthetic sorbitol-6-phosphate was only slightly hydrolyzed under the same experimental conditions. In the other experiments, it was shown that F-6-P reductase catalyzes only the oxidation of M-1-P but not of sorbitol-6-phosphate (see the next "substrate and coenzyme specificity"). These experimental results indicate that the reaction product of F-6-P reduction is M-1-P.

Substrate and Coenzyme Specificity—The F-6-P reductase preparation catalyzes the reduction of F-6-P in the presence of DPNH, G-6-P was also reduced by the same enzyme preparation. When only G-6-P was incubated with crude extracts of *P. oryzae*, the increase of F-6-P was observed by Roe reaction, and one thirds of G-6-P was converted to F-6-P at an equilibrium. Therefore the G-6-P reduction by the F-6-P reductase preparation is possibly due to a contamination with phosphohexoisomerase of the enzyme preparation. Neither free fructose nor glucose was reduced by the same enzyme preparation. This enzyme preparation oxidized synthetic M-1-P in the presence of DPN, which could not be replaced by TPN. Sorbitol-6-phosphate, dulcitol-6-phosphate and free mannitol were not oxidized.

Properties of Enzyme—As shown in Fig. 2 the optimal pH for M-1-P oxidation was found to be 9.5. PCMB ($10^{-4}\,M$) caused 70 per cent inhibition, which could be partially restored by the addition of cysteine. NH_2OH, semicarbazide and NaF at $10^{-2}M$ had no effect on the reaction. EDTA, NaN_3, or monoiodoacetate at $10^{-3}M$ also had no effect. The Michaelis constants were $5\times10^{-5}M$ for M-1-P, and $7\times10^{-5}M$ for DPN.

M-1-Pase

Assay—In a small test tube 85 μmoles of veronal-acetate buffer, pH 8.0, 10 μmoles of $MgCl_2$ and a suitable amount of enzyme solution were placed in a total volume of 1.9 ml. and the reaction was started by the addition of 10 μmoles of M-1-P in 0.1 ml. After incubation at 28°C for 30 minutes, the reaction was stopped by adding 0.3 ml. of 60% $HClO_4$ to each tube. The resulting precipitate was removed by filtration. From the filtrate 1.15 ml. of aliquot was withdrawn for the determination of inorganic phosphate. The same mixture containing no substrate was taken as a blank. One unit of this enzyme activity was defined as the amount of enzyme that caused the liberation of 1 μmole of inorganic phosphate under the assay conditions. The amount of liberated inorganic phosphate was proportional to the enzyme concentration within the range up to 2 units of enzyme under standard assay conditions. Specific activity was expressed as units per mg. of protein.

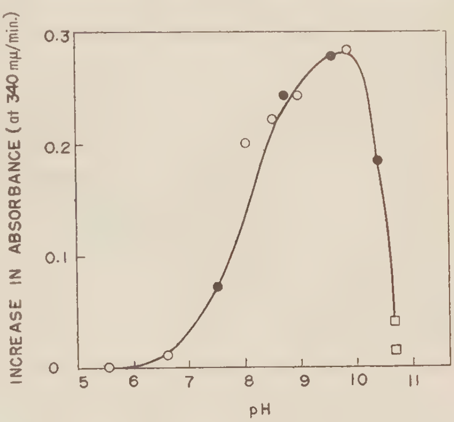

FIG. 2. Effect of pH on F-6-P reductase.

In standard assay system, the buffer was replaced by the same amount of indicated buffer.
—○—: Acetate, —●—: Glycine,
—□—: Carbonate.

Enzyme Preparation—Cell-free extracts (specific activity: 3.3) were treated with $MnCl_2$ solution in the same manner as for F-6-P reductase. To 20 ml. of this manganese supernate 4.9 g. of ammonium sulfate was added and the precipitate was centrifuged off at 12,000×*g* for 5 minutes. To the supernate 6.4 g. of ammonium sulfate was added and centrifuged at 14,000×*g* for 15 minutes.

The supernatant fluid was discarded and the precipitate was dissolved in 5 ml. of water (specific activity: 6.0, yield of the activity: 66 per cent of crude extract). This enzyme was rather unstable in the presence of ammonium sulfate, and the prolonged contact with higher concentration of this salt caused complete inactivation.

Stoichiometry—In a test tube 3.0 ml. of water and 0.2 ml. of M-1-Pase solution were placed. The reaction was initiated by adding 80 μmoles of M-1-P in 0.8 ml., adjusted to pH 7.0. The mixture was incubated at 28°C for 60 minutes then the tube was heated in a boiling water bath for 5 minutes. Another tube, which was taken as a control, was heated before the addition of M-1-P solution. After removal of denatured protein by centrifugation the supernate was subjected to assay for M-1-P using F-6-P reductase, and for inorganic phosphate. To 0.3 ml. of this supernate 0.3ml. of 25% barium acetate solution and 2.4 ml. of 95% ethanol were added and the resulting insoluble barium salt was centrifuged off. This barium-ethanol supernate was analyzed for free mannitol. The results are presented in Table III, in which the disappearance of M-1-P was well balanced with the formation of inorganic phosphate and of free mannitol.

TABLE III

Stoichiometric Studies of M-1-Pase

−ΔM-1-P (μmoles)	ΔInorganic phosphate (μmoles)	ΔMannitol (μmoles)
44	46	51

Experimental conditions are described in the text.

Substrate Specificity—Several polyol phosphates and sugar phosphates were incubated with M-1-Pase preparation and the liberation of inorganic phosphate was determined. The results are summarized in Table IV. Phosphate compounds other than M-1-P proved to be not hydrolyzed by this enzyme preparation.

Properties of Enzyme—The optimal pH for the hydrolysis of M-1-P by this enzyme preparation was found to lie near 7.0 (Fig. 3).

TABLE IV

Substrate Specificity of M-1-Pase

Substrate	Inorganic phosphate liberated (μmoles)
Mannitol-1-phosphate	1.28
Sorbitol-6-phosphate	0.01
Dulcitol-6-phosphate	0.02
α-Glycerophosphate	0.01
Glucose-1-phosphate	0.00
Glucose-6-phosphate	0.03
Fructose-6-phosphate	0.02
Fructose-1,6-diphosphate	0.00

Standard assay conditions were used except that 10 μmoles of indicated substrate were added in place of M-1-P.

The reaction was stimulated in the presence of several divalent ions (Table V). PCMB ($5\times10^{-4}M$) and EDTA ($5\times10^{-3}M$) caused complete inhibition, $5\times10^{-3}\,M$ NaF or NaCN was slightly inhibitory. The Michaelis constant was $3\times10^{-3}\,M$.

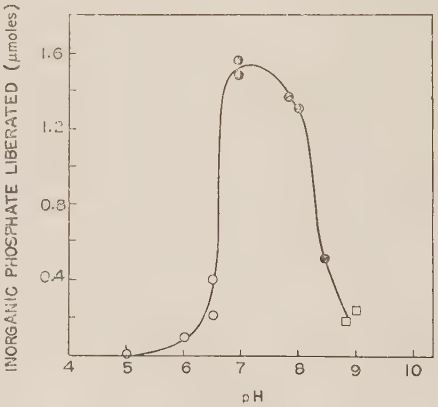

FIG. 3. Effect of pH on M-1-Pase activity. In standard assay system, the buffer was replaced by the same amount of indicated buffer. —○—: Acetate, —●—: Glycine, —□—: Carbonate.

Table V

Effect of Metals on M-1-Pase Reaction

Addition	Inorganic phosphate liberated (μmoles)
None	0.37
$MgCl_2$	0.69
$MnCl_2$	0.78
$FeSO_4$	0.93
$FeCl_3$	0.26
$CoCl_2$	0.38
$NiCl_2$	0.05
$CaCl_2$	0.07
$CuSO_4$	0.01
$ZnSO_4$	0.05
$BaCl_2$	0.21

Standard assay conditions were used except that 10 μmoles of indicated salt were added in place of $MgCl_2$.

Activities of the Two Enzymes at Different Growth Stages of the Fungus

Cell-free extracts were prepared from the mycelia of *P. oryzae* at different growth stages and assayed for F-6-P reductase and M-1-Pase respectively. The results are shown in Fig. 4.

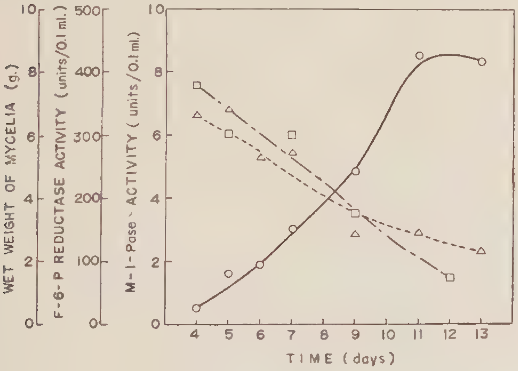

Fig. 4. Activities of F-6-P reductase and M-1-Pase in the extracts of *P. oryzae* at different growth stages. Standard assay conditions were used.

—○— Growth, --□-- F-6-P reductase, --△-- M-1-Pase.

Occurrence of the Two Enzymes

Activities of F-6-P reductase and of M-1-Pase were investigated in the cell-free extracts of two strains of *Aspergillus* and two strains of *Neurospora*. The results are presented in Table VI. To distinguish M-1-Pase activity from non-specific phosphatase, liberations of inorganic phosphate from both M-1-P and G-6-P were determined separately. In each case M-1-P was hydrolyzed about ten times as fast as G-6-P, and the presences of M-1-Pase in these extracts were demonstrated.

Table VI

Distribution of F-6-P Reductase and M-1-Pase

Organism	F-6-P reductase activity[1]	Inorganic phosphate liberated from[2]	
		M-1-P	G-6-P
Aspergillus oryzae	45	0.93	0.12
Aspergillus niger	57	1.30	0.12
Neurospora crassa	4	1.48	0.14
Neurospora sitophila	−3	1.98	0.10

1) Units per 0.1 ml. of extracts under standard assay conditions.

2) μmoles per 0.3ml. of extracts under standard assay conditions in which M-1-P or G-6-P was added as a substrate.

DISCUSSION

The conversion of hexose to mannitol has been known to be catalyzed by polyol dehydrogenase, which catalyzes the reversible dehydrogenation of polyol to sugar in the presence of pyridine coenzymes. An alternative pathway, the conversion at a level of phosphorylated compounds has been reported recently (*3*, *14*, *15*). In 1954 Racker reported the enzymic reduction of F-6-P in *Escherichia coli* extracts and assumed hexitol phosphate as a reaction product (*14*). Later F-6-P reductase of *E. coli* (*3*) and of *Diplococcus pneumoniae* (*15*) were purified and the reaction product was identified as M-1-P.

In cell-free extracts of *P. oryzae* no polyol dehydrogenase activity was detectable, but F-6-P reductase and M-1-Pase were demonstrated. The conversion of sugar to mannitol in this fungus therefore takes place only at the level of phosphorylated compounds, and the pathway of the formation of mannitol can be written as follows:

$$\text{F-6-P} \overset{\text{DPNH}}{\rightleftharpoons} \text{M-1-P} \longrightarrow \text{Mannitol}$$

The equilibrium of the reversible dehydrogenation can be favoured far to the side of M-1-P formation by the action of specific phosphatase. Reduction at the level of phosphorylated compounds may, therefore, be more advantageous to the accumulation of mannitol.

Both F-6-P reductase and M-1-Pase were also found in the crude extracts of *A. oryzae* and *A. niger*, and in *N. crassa* and *N. sitophila* M-1-Pase was demonstrated but not F-6-P reductase. It is a very interesting problem to know whether M-1-P may be produced in these organisms by the alternative pathway different from F-6-P reduction.

The accumulation of mannitol would have two different biological significances in microorganisms. One is as a reserve material and the other as a fermentation product. In the case of *P. oryzae* the content of mannitol in the mycelia showed no significant change throughout the whole stage of growth. Therefore it is more reasonable to consider that mannitol is accumulated as a fermentation product and not as a reserve material in this fungus.

SUMMARY

1. Mannitol of about 10 per cent of dry weight of mycelia was accumulated in *Piricularia oryzae* through the whole stage of growth on sucrose medium.

2. Cell-free extract of *P. oryzae* was found to catalyze the reduction of F-6-P in the presence of DPNH, followed by the hydrolysis of the reaction product.

3. The enzyme which catalyzes the reduction of F-6-P was purified and its reaction product was characterized as M-1-P. Properties of this enzyme were investigated.

4. The enzyme which hydrolyzes M-1-P was found to be highly specific to M-1-P. Properties of this enzyme was also examined.

5. In *P. oryzae* the pathway of the formation of mannitol can be written as:

$$\text{F-6-P} \overset{\text{DPNH}}{\rightleftharpoons} \text{M-1-P} \longrightarrow \text{Mannitol}$$

6. The activities of these two enzymes were highest at very early stage of growth of this fungus.

7. Significance of the accumulation of mannitol in *P. oryzae* was discussed.

8. Both F-6-P reductase and M-1-Pase were demonstrated in *Aspergillus oryzae* and *Aspergillus niger*. In *Neurospora crassa* and *Neurospora sitophila* only M-1-Pase was found.

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Incorporation of Amino Acids into Cytochrome c in a Bacterial Cell-Free System

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Previously, we reported some properties of a bacterial cell-free system from *Pseudomonas-P* which actively incorporated amino acids into proteins (*1*). In order to determine whether actual protein synthesis occurred in this system the isolation of a well-characterized radioactive protein after the incorporation of labelled amino acids would be required.

Cytochrome c was chosen as a specific protein, because it was found that this organism, under suitable conditions, synthesized a large amount of cytochrome c which was mainly located in the cytoplasmic membrane fraction.

In this paper, incorporation of radioactive amino acids into the proteins of the cell-free system, and isolation of radioactive cytochrome c will be described.

EXPERIMENTALS

Microorganism—*Psedomonas-P* (*3*) was grown in pepton-bouillon medium for 16 hours at 30°C under aerobic conditions, and 1.2 ml. of this culture was transferred to 150 ml. of fresh medium and grown for another 5 hours, under the same conditions. The cells were then collected by centrifugation and washed twice with distilled water. At this phase of growth the cells synthesize cytochromes most actively (*2*).

Cell-Free Preparation—The cells (500 mg. wet weight) were treated with lysozyme (3 mg., Nutritional Biochemicals Corp., Cleveland, Ohio, U. S. A. or Worthington Biochemical Corp. New Jersey, U. S. A.) in 30 ml. of tris hydroxymethyl aminomethane (Tris) buffer (0.05 M, pH 8.0) containing sodium ethylenediamine tetraacetate (85 μmoles) and potassium chloride (15 mmoles). The cells were converted to protoplasts after 30 minutes incubation at 30°C, and the protoplasts were collected by centrifugation and homogenized in 10 ml. of magnesium sulphate solution (0.05 M, pH 7.4) containing deoxyribonuclease (100 μg., Sharp & Dohm, West point, Pa. U.S.A.). The cell membrane fraction was obtained by centrifugation at 20,000 × g for 20 minutes, and resuspended either in Tris buffer (0.05 M, pH 7.4) containing magnesium sulphate (0.05 M) or Tris buffer (0.05 M, pH 7.4) without magnesium ions. Hereafter, these two preparations will be called MgP and WP, respectively. The details of this procedure and some characteristics of these preparations were described in the previous report (*1*).

Amino Acid Incorporation—The reaction mixture containing the cell-free preparation, phosphate, pepton (Polypepton, Takeda Pharm. Co., Osaka) and radioactive amino acids mixture (C^{14}algal protein hydrolysate) was incubated at 30°C with shaking. Details are described in the legend of Fig. 1. One ml. aliquots of the reaction mixture were taken at time 0, 30 and 60 minutes, and treated in the cold with 10% trichloroacetic acid (TCA) containing 1% casein hydrolysate. The precipitate was extracted with hot TCA (5 per cent) and ethanol-ether (1 : 1) and the radioactivity of the residual protein fraction was measured as previously described (*1*).

Extraction and Purification of Cytochrome c—The procedure is similar to that described by Horio (*4*). After 60 minutes, the reaction mixture was poured into ten times its volume of acetone (−20°C) and stirred for 30 minutes at −10°C. The precipitates were collected by centrifugation, and cytochromes were extracted three times with sodium citrate (0.1 M, pH 6.5). The extracted cytochrome c was purified by negative adsorption on Amberlite XE-64 cation exchanger, followed by ammonium sulfate fractionation. The precipitate occurring between 35 and 95% ammonium sulfate saturation was collected and dissolved in ammonium phosphate buffer (0.1 M, pH 6.0) and dialysed against the same buffer. Three mg. of non-radioactive cytochrome c preparation obtained by the same purification procedure was added as a carrier. This mixture was transferred on an alumina-oxide column buffered with ammonium phosphate buffer. After the impurities were washed out of the column, the buffer was changed to potassium phosphate buffer

(0.5 *M*, pH 7.4), and cytochrome c_{554} was eluted as a single band of reddish colour.

RESULTS

Amino Acid Incorporation into Protein Fraction—It will be seen in Fig. 1 that the incorporation of radioactive amino acids into protein fraction by MgP proceeded continuously for 60 minutes. The activity of WP, however, was less than 4 per cent of that of MgP.

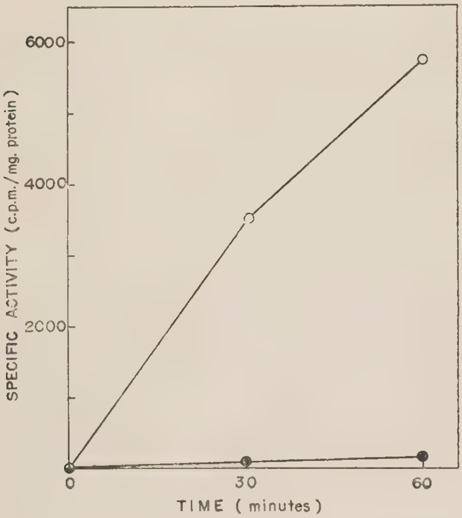

FIG. 1. Amino acid incorporation into protein fraction of MgP and WP.

Reaction mixture contained 11.5 mg. (protein) of preparation, C^{14} amino acid mixture (3×10^5 c.p.m.), 5 mg. of peptone and 0.2 mmole of phosphate buffer (pH 7.2). Final volume was 15 ml. Incubation was carried out aerobically at 30°C.
—○— MgP, —●— WP

Isolation of Radioactive Cytochrome c—Chromatographic patterns of radioactivity, protein concentration and 419 mμ absorption are shown in Fig. 2. As can be seen, two separate peaks were obtained by chromatography. Peak I contained an appreciable amount of protein. The absorption at 419 mμ, however, was much less than peak II. It is worthy to note that the pattern of protein concentration of peak II followed very well those of 419 mμ absorption and radioactivity. Peak II was identified as cytochrome c_{554} from its characteristic absorption in visible region (Fig. 3).

The coincidence of radioactivity peak with the cytochrome c_{554} peak (Fig. 2) demonstrated that C^{14} labelled cytochrome c_{554} had been formed. Radioactivity recovered in this fraction amounted to approximately 5 per cent of that incorporated into the protein fraction of MgP.

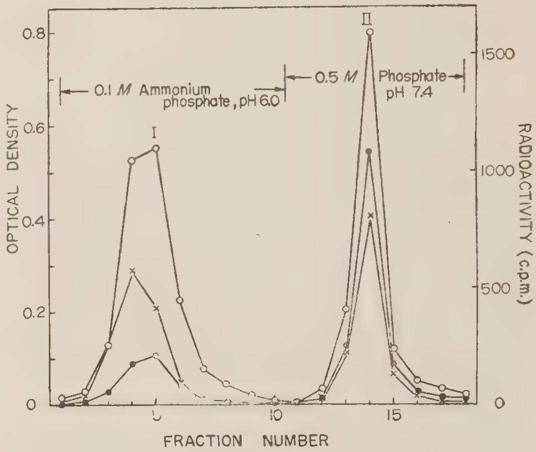

FIG. 2. Chromatographic patterns of radioactivity, protein concentration and 419 mμ absorption.

—○— Protein (Folin-phenol. 700 mμ absorption)
—●— 419 mμ absorption
—×— radioactivity (c. p. m.)

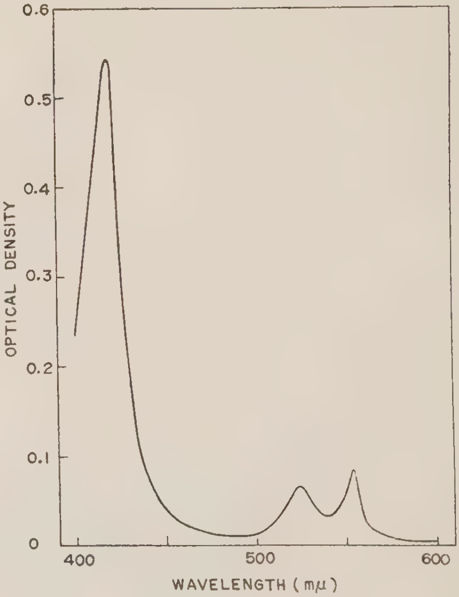

FIG. 3. Absorption spectrum of peak II fraction.

DISCUSSION

Simpson *et al.* (*5*) reported the net synthesis of cytochrome c by calf-heart mitochondria, and C^{14}-lysine and C^{14}-valine were shown to be incorporated into the molecule of cytochrome c.

Schweet *et al.* (*6*) isolated the radioactive hemoglobin after the incubation of rabbit microsomes with C^{14}-leucine. It was shown by IRC-50 chromatography that the radioactivity was associated with the hemoglobin fraction.

In the present experiment amino acid incorporation into cytochrome c by the cell-free preparation was described. In this kind of experiment careful measure should be taken for the contribution of residual intact cells. The effect of magnesium concentration on the nature of the preparation was described previously (*1*). In the present experiment, WP was used as a control preparation, and as shown in Fig. 1., radioactivity incorporated into the protein fraction of WP was less than 4 per cent of that of MgP, and radioactivity was not detectable in the cytochrome c_{554} isolated from WP. These results ruled out any contribution of residual intact cells to the incorporation of amino acids into cytochrome c by the active cell-free preparation.

The present state of uncertainty about the extraction yield and the purity of cytochrome c_{554}, makes it difficult to carry out experiments quantitatively. However, the evidence presented strongly favors the view that the isolated cytoplasmic membrane preparation can incorporate amino acids into cytochrome c_{554}.

SUMMARY

A cell-free preparation from crushed protoplasts of *Pseudomonas-P* incorporated C^{14}-labelled amino acids actively into protein fraction.

C^{14}-labelled cytochrome c_{554} was isolated and characterized from the reaction mixture.

The authors thanks Prof. B. Maruo, and Dr. H. Takahashi of this Institute for their advice during this investigation. Thanks are due to Miss. E. Yoshida for her co-operation and valuable discussions throughout this work.

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Activation of *Pseudomonas* Cytochrome Oxidase by Catalase

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From an acetone powder of *Pseudomonas aeruginosa*, *Pseudomonas* (*P*)-cytochrome oxidase has been extracted and purified by chromatography using an ion exchange resin and by zone electrophoresis. It was obtained in an almost pure form as judged by ultracentrifugal analysis (*1–5*). The cytochrome oxidase shows absorption maxima at 630 and 625 mμ in its oxidized and reduced forms, respectively. The absorption peaks in the red region have been thought to be those of "cytochrome a_2". However, the *P*-cytochrome oxidase has a c-type haem besides a haem a_2 in its molecule. Therefore, this cytochrome oxidase is not a simple cytochrome a_2 with only a haem a_2 in the molecule.

Unlike animal cytochrome a (*6*), *P*-cytochrome oxidase dissolves in water without the aid of any detergent such as cholate. Its high solubility in water is due to the hydrophilic property of the haem a_2 (*4*, *5*). Animal cytochrome a functions as a cytochrome oxidase only in cooperation with cytochrome c (*7*), whereas *P*-cytochrome oxidase, having two different haems in a single molecule, oxidizes hydroquinone, ascorbate, reduced *P*-cytochrome c-551 (*1*, *2*, *8*, *9*) and reduced *P*-blue protein (*1*, *2*). It also acts as a nitrite reductase under anaerobic conditions (*10*).

During studies on *P*-cytochrome oxidase, it was found that the rate of oxidation of its substrates decreased rapidly after the reaction had begun, but that the presence of catalase could prevent this phenomenon. It was also found that a crude sample of the cytochrome oxidase showed a high catalase activity. These findings suggest that in the living cells of the organism the cytochrome oxidase may function in cooperation with catalase. To study this possibility, the effect of catalase on the oxidation of various substrates by the cytochrome oxidase were examined. The results are reported in the present paper.

MATERIALS AND METHODS

P-cytochrome oxidase and *P*-cytochrome c-551 were purified to about 70 per cent purity from the acetone powder of cells of *Pseudomonas aeruginosa* according to the method of Horio (*1*, *2*) and Horio *et al.* (*3–5*, *8*, *9*). Bovine catalase was crystallized according to the method of Kitagawa and Shirakawa (*11*). Yeast lactate dehydrogenase was purified according to the method of Yamanaka *et al.* (*12*) as slightly modified by Yamashita (*13*). Bacterial α-amylase was crystallized according to the method of Hagihara (*14*). Glucose oxidase was obtained in a crystalline form from the culture medium of *Penicillium amagasakiense* according to the method of Kusai *et al.* (*15*).

Oxygen consumption was determined in a Warburg manometer at 37°C. The reaction was started after a 5 minute equilibration period. Optical densities were determined using a Shimadzu photoelectric spectrophotometer, Type QB-50, at room temperature. The dry weight of protein in the crude enzyme solution was estimated by precipitation with trichloroacetic acid according to the methd of Higashi (*16*). Zone electrophoresis was carried out on a starch column in 0.067 *M* phosphate buffer of pH 7.0 in a refrigerator (5°C) with frequent adjustment of amperage to 20 mA. Catalase activity was determined according to the method of Bonnichsen *et al.* (*17*), and its concentration was determined from its extinction assuming that the millimolar extinction at 280 mμ is 280 mM^{-1}cm.$^{-1}$ (*17*).

RESULTS

Activating Effect of Catalase on the Oxidation

of Ascorbate and Hydroquinone Catalysed by P-Cytochrome Oxidase—The oxidation velocities of ascorbate and hydroquinone catalysed by *P*-cytochrome oxidase decreased rapidly as the reactions proceeded, as shown in Fig. 1.

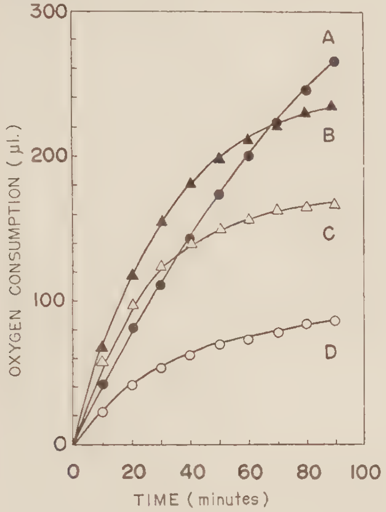

Fig. 1. Activating effect of catalase on oxidation of ascorbate and hydroquinone, at pH 6.4 and 37°C.

The reaction mixture consisted of 0.05 *M* substrate, 0.05 *M* phosphate buffer, 9.1×10^{-6} *M* *P*-cytochrome oxidase, with and without 0.80×10^{-6} *M* catalase, in a total volume of 2.0 ml. Reactions were started by addition of substrate. (A) Ascorbate+*P*-cytochrome oxidase+catalase, (B) hydroquinone + *P*-cytochrome oxidase + catalase, (C) hydroquinone+*P*-cytochrome oxidase and (D) ascorbate+*P*-cytochrome oxidase. With ascorbate, 5×10^{-3} *M* ethylenediaminetetraacetate was also added.

The decrease of the rate in experiments with ascorbate was apparently more rapid than with hydroquinone. As the substrates were added in an excess, these phenomena can not be due to the exhaustion of the substrates. Thus, it is to be assumed that the reaction product may inhibit the cytochrome oxidase. As the matter of fact, *p*-benzoquinone, which is a probable product of the oxidation of hydroquinone, inhibited about 15 per cent of the oxidation of the substrate at a concentration of 5×10^{-3}*M*. The concentration of *p*-benzoquinone used was equivalent to 112 μl. of oxygen consumption as described below. However, the decrease in oxygen consumption described above occurred even when 40 μl. of oxygen had been consumed. By addition of catalase, the oxygen consumption was much accelerated and the rate continued almost linearly for a considerable period with either substrate. The oxidation of ascorbate was accelerated more than that of hydroquinone by catalase. Bacterial α-amylase and gelatin did not replace catalase (Fig. 2). Furthermore, partially inactivated catalase, obtained by prolonged dialysis against distilled water, and having 25 per cent of the original enzyme activity, had less activating effect than the original catalase, when added at the same concentration as judged from the optical density at 280 mμ (Fig. 3). When reduced *P*-cytochrome c-551 was used as an electron donor, catalase had no activat-

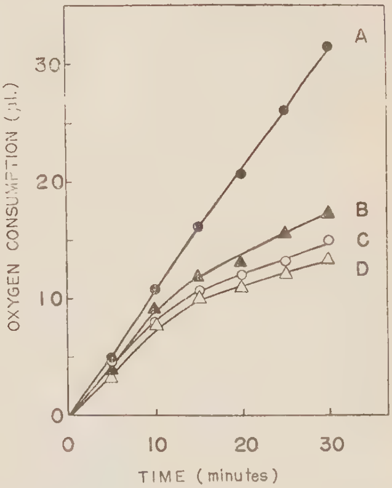

Fig. 2. Effect of gelatin and bacterial α-amylase on the oxidation of ascorbate.

The reaction mixture contained 0.05 *M* ascorbate, 2.6×10^{-6} *M* *P*-cytochrome oxidase, 0.05 *M* phosphate buffer, and 5×10^{-3} *M* ethylenediaminetetraacetate, besides the protein tested. Reactions were started by addition of ascorbate at pH 6.4 and 37°C. The total volume was 2.0 ml. (A) Ascorbate + *P*-cytochrome oxidase + 0.012% catalase, (B) ascorbate+*P*-cytochrome oxidase+0.024% bacterial α-amylase, (C) ascorbate+*P*-cytochrome oxidase and (D) ascorbate+*P*-cytochrome oxidase+0.1% gelatin.

ing effect (Fig. 4). The activating effect was not observed also from manometrical measurements using yeast lactate dehydrogenase, lactate and *P*-cytochrome c-551 as an electron donating system (Fig. 5).

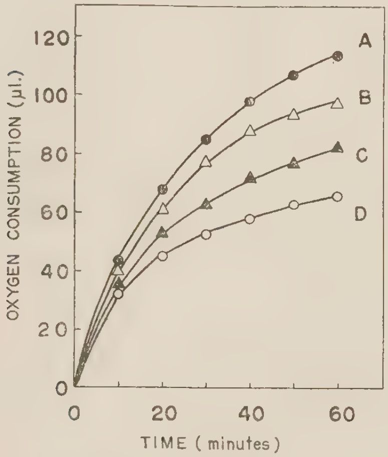

FIG. 3. Effect of partially inactivated catalase on *P*-cytochrome oxidase.

A partially inactivated catalase was prepared by dialysis of a catalase preparation against distilled water for 3 days. The inactivated catalase had 25 per cent of the original enzyme activity as judged from the first order reaction constant for decomposition of hydrogen peroxide.

The reaction mixture consisted of 0.05 *M* ascorbate, 5×10^{-3} *M*ethylenediaminetetraacetate, 0.05 *M* phosphate buffer, and 4.7×10^{-6} *M P*-cytochrome oxidase, besides catalase, in a total volume of 2.0 ml. Reactions were started by addition of ascorbate and carried out at pH 6.4 and 37°C. (A) $+1.9\times10^{-6}$ *M* catalase, (B) $+0.95\times10^{-6}$ *M* catalase, (C) + inactivated catalase corresponding to 1.9×10^{-6} *M*, and (D) control.

The Effect of Hydrogen Peroxide on the Oxidation of Hydroquinone Catalysed by P-Cytochrome Oxidase—If glucose and glucose oxidase was added to reaction mixture as a hydrogen peroxide generating system, the oxygen consumption was much reduced in the absence of catalase. On addition of catalase, the oxygen consumption was restored almost to the original level (Fig. 6). The activating effect of catalase, however, was observed only when catalase was added at the beginning of the reaction. Once the rate of oxygen consumption had been reduced, it could not be restored to its original rate by addition of catalase. The oxygen consumption on the addition of glucose oxidase and glucose was about 2 μl. during a 10 minute period. Therefore, the maximum hydrogen peroxide formed was only 0.09 μmoles per vessel. Exogenous addition of hydrogen peroxide from the side arm of the vessels inhibited 15 and 50 per cent of the oxidation of hydroquinone in concentrations of 10^{-5} and 10^{-4} *M*, respectively, during the reaction. As the volume of reaction mixture per vessel was 2.0 ml., these concentrations of hydrogen peroxide corresponded to 0.02 and 0.2 μmoles, respectively. Hydrogen peroxide continuously produced by an enzymatic system is apparently more effective than exogenously added reagent in inhibiting the reaction. This means that the substrate reduces

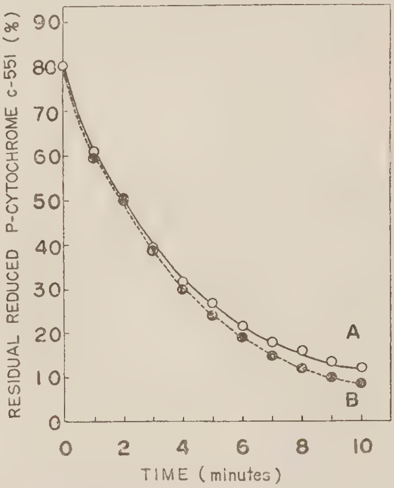

FIG. 4. Effect of catalase on oxidation of reduced *P*-cytochrome c-551.

The oxidation of reduced *P*-cytochrome c-551 catalysed by *P*-cytochrome oxidase was determined by the decrease in optical density at 551 mμ at pH 5.0 and 11°C, under aerobic conditions. Reactions were started by addition of the cytochrome oxidase.

The reaction mixture contained 2.5×10^{-5} *M* reduced *P*-cytochrome c-551, 1.5×10^{-6} *M P*-cytochrome oxidase and 0.072 *M* phosphate buffer, besides catalase, in a total volume of 2.8 ml. (A) $+0.53\times10^{-6}$ *M* catalase, (B) no catalase.

the hydrogen peroxide before the latter has reacted with the cytochrome oxidase in the reaction mixture.

The oxygen consumed in the oxidation of hydroquinone corresponded to the following formula;

$$\text{hydroquinone}+\tfrac{1}{2}\,O_2=p\text{-benzoquinone}+H_2O,$$

when a definite amount of hydroquinone was oxidized in the presence of excess cytochrome oxidase for a prolonged period. This, however, does not exclude the possibility that hydrogen peroxide was formed as a direct reaction product, because hydroquinone will consume hydrogen peroxide as mentioned above, and the inactivated *P*-cytochrome oxidase will decompose hydrogen peroxide as described below.

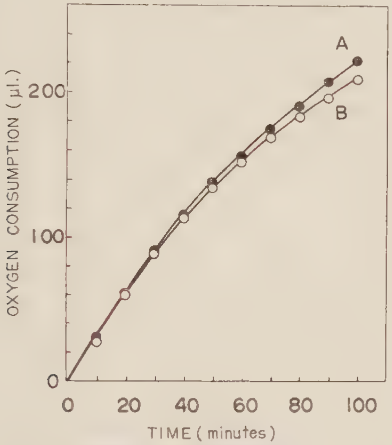

Fig. 5. Effect of catalase on the oxidation of *P*-cytochrome c-551 continuously reduced by lactate and yeast lactate dehydrogenase, at pH 6.4 and 28°C.

The reaction mixture consisted of 0.05 *M* lactate, 7.1×10^{-6} *M* *P*-cytochrome c-551, 4.7×10^{-6} *M* *P*-cytochrome oxidase, yeast lactate dehydrogenase with and without 0.13×10^{-6} *M* catalase. Reactions were started by addition of the cytochrome oxidase. The time required for the lactate dehydrogenase to decolourize methylene blue was 3 seconds (*12*). (A) +Catalase, (B) no catalase.

Catalase Activity Found in the Crude P-Cytochrome Oxidase Sample—The crude *P*-cytochrome oxidase, which has a strong catalase activity, oxidized ascorbate and hydroquinone linearly for a considerable period. As the cytochrome oxidase became more purified, its catalase activity became weaker (Table I), and the rate of oxygen consumption catalysed by the sample lost this linearity. The purest sample obtained still showed a considerable catalase activity, but it appeared to be non-enzymatic, because, in this case, hydrogen peroxide in the reaction mixture was consumed rapidly in an initial period and then the reaction practically ceased, in measuring catalase activity. Since *P*-cyto-

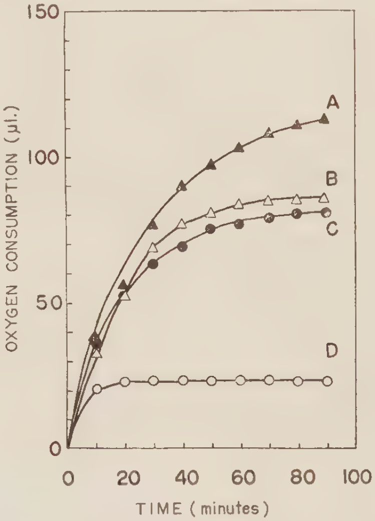

Fig. 6. Inactivation of *P*-cytochrome oxidase by glucose and glucose oxidase, and its reactivation by catalase.

The reaction mixture contained 4.7×10^{-6} *M* *P*-cytochrome oxidase, 0.05 *M* hydroquinone and 0.05 *M* phosphate buffer, besides glucose, glucose oxidase and catalase, in a total volume of 2.0 ml. The concentrations of glucose, glucose oxidase and catalase were 0.01 *M*, *ca.* 10^{-9} *M* and 1.6×10^{-6} *M*, respectively. (A) Hydroquinone+*P*-cytochrome oxidase+catalase, (B) hydroquinone+*P*-cytochrome oxidase+glucose+glucose oxidase+catalase, (C) hydroquinone+*P*-cytochrome oxidase, and (D) hydroquinone+*P*-cytochrome oxidase+glucose+glucose oxidase.

The oxygen consumption on oxidation of glucose catalysed by glucose oxidase was 7 µl. during the first 30 minutes. Reactions were started by addition of hydroquinone and glucose, and carried out at pH 6.4 and 37°C.

TABLE I

Catalase Activity of P-Cytochrome Oxidase Preparations at Various Stages of Purification

	k (min.$^{-1}$)/g. protein[1)]
Extract of acetone powder (after rivanol treatment) (*4*)	1630
Eluate from Duolite CS-101	43
After electrophoresis (first time)	8
After electrophoresis (third time)	52

1) k is the first order reaction constant in the measurement of catalase activity.

chrome oxidase is labile in the presence of hydrogen peroxide, especially in its purified state, it is inferred from the above results that the *P*-cytochrome oxidase is easily destroyed during measurement of catalase activity, and that hydrogen peroxide is consumed during the decomposition of the cytochrome oxidase. Thus, it is reasonable to conclude that the cytochrome oxidase is destroyed, during the oxidation of substrates, by the small amount of hydrogen peroxide formed as a direct reaction product. Although the values shown in the Table may vary with the conditions under which the organism is cultivated, it is certain that the catalase activity is removed from the *P*-cytochrome oxidase preparation during the purification procedure.

DISCUSSION

As reported already, *P*-cytochrome oxidase has the general properties of a cytochrome oxidase, whereas animal cytochrome a functions only in cooperation with cytochrome c. *P*-Cytochrome oxidase also acts as a nitrite reductase under anaerobic conditions.

The oxidation velocities of ascorbate and hydroquinone catalysed by *P*-cytochrome oxidase decreased rapidly during the reaction period, as the enzyme became more purified, even though excess substrate was present in the reaction mixture. These findings suggest that the reaction products may inhibit the enzyme. However, *p*-benzoquinone, a probable product of the reaction did not inhibit the *P*-cytochrome oxidase, except in a considerably higher concentration than could be expected to be formed during the reaction. Since the addition of catalase eliminated the decrease in rate of oxidation described above, it seems likely that the inhibition of *P*-cytochrome oxidase during the reaction is due to the action of hydrogen peroxide as a reaction product.

Frieden and Maggiolo (*18*) showed that ascorbic acid oxidase was activated by catalase and that the same activation was obtained with edestin, albumin, pepsin, chymotrypsin, β-lactoglobulin and gelatin. Therefore, the activating effect found in the case of ascorbic acid oxidase was not unique for catalase.

On the other hand, gelatin and bacterial α-amylase showed no activating effect on *P*-cytochrome oxidase. Moreover, partially inactivated catalase had less effect. Therefore, the activating effect of catalase on *P*-cytochrome oxidase can be said to be due to a unique property of catalase. When glucose oxidase were added as a continuously hydrogen peroxide generating system, the oxidation velocity of the substrate catalysed by *P*-cytochrome oxidase decreased. However, the addition of catalase almost completely eliminated the effect of addition of glucose and glucose oxidase. These facts lead us to the conclusion that *P*-cytochrome oxidase is inactivated by the hydrogen peroxide produced during the oxidation of the substrates. The activating effect of catalase was observed more strikingly in the oxidation of ascorbate than of hydroquinone. This may suggest that the ratio of hydrogen peroxide produced to oxygen consumed varies with the substrate. As already indicated, the stoichiometry of the reaction obtained from oxygen consumption when the substrate had been exhausted does not conflict with the formation of hydrogen peroxide as a direct reaction product.

An extract of an acetone powder of *Pseudomonas aeruginosa* showed strong catalase activity. As described above, on purification of the *P*-cytochrome oxidase, the catalase activity in the preparation decreased. From these results, it may be that in the living cells, *P*-cytochrome oxidase functions fully only in cooperation with catalase. However, simply from the fact that the two enzymes are present in the same extract, it can not be concluded that they are localized in the same area in the cells.

P-cytochrome c-551 is thought to function in the same manner in the cells of the organism as cytochrome c does in animal cells (*19*). Reduced *P*-cytochrome c-551, therefore, is biologically a direct electron donor to *P*-cytochrome oxidase. Spectrophotometrically, the oxidation of reduced *P*-cytochrome c-551 was not found to be affected by catalase. On spectrophotometrical measurement, however, only a little oxygen is consumed and then the hydrogen peroxide produced, if formed, is too little to affect the reaction to any measurable extent. The oxidation of *P*-cytochrome c-551 reduced by yeast lactate dehydrogenase can be measured manometrically in the presence of *P*-cytochrome oxidase. However, there is no apparent activating effect of catalase. These facts suggest that the activating effect of catalase on the *P*-cytochrome oxidase has no biological significance in this organism. However, it may be that some enzyme such as D-amino acid oxidase which produces hydrogen peroxide is present close to *P*-cytochrome oxidase in a very restricted area of the cells, and that catalase is necessary to protect the cytochrome oxidase from this hydrogen peroxide (*20*).

Apart from its biological significance, it is important that hydrogen peroxide is produced during the oxidation of ascorbate or hydroquinone but not during that of reduced *P*-cytochrome c-551. If electrons liberated from a substrate react with oxygen only after they are accepted by the *P*-cytochrome oxidase, the reduction product of oxygen should be the same kind of substance irrespective of the nature of the substrate. Although when reduced *P*-cytochrome c-551 is used as a substrate, it may be assumed that the hydrogen peroxide, once produced, is destroyed by the catalytic activity of the cytochrome, the cytochrome has no catalase activity. From the facts and inferences described above, it may be concluded at least in the oxidation reaction catalysed by *P*-cytochrome oxidase, that three components, oxygen, an electron donor and the enzyme, should react simultaneously.

SUMMARY

The rate of oxidation of ascorbate and hydroquinone catalysed by a purified *Pseudomonas* cytochrome oxidase decreased rapidly during the reaction period. On addition of catalase, this decrease was eliminated and oxygen consumption was accelerated, 3-fold with ascorbate and 1.5-fold with hydroquinone. It is concluded that *P*-cytochrome oxidase was inactivated during the reaction by hydrogen peroxide formed as a direct reaction product. When reduced *P*-cytochrome c-551 was used as an electron donor, however, no activating effect of catalase was observed. Therefore, the activation effect of catalase on *P*-cytochrome oxidase may be biologically insignificant. However, the fact that hydrogen peroxide was produced on the oxidation of ascorbate and hydroquinone but not on the oxidation of reduced *P*-cytochrome c-551 indicates that the reaction mechanism differs with different electron donors.

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On the Mechanism of Anaerobic Formation of Succinate by *Proteus vulgaris*

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As reported in the previous paper (*1*), a considerable amount of succinate was produced from glucose under anaerobic condition by *Proteus vulgaris*. It is a long-established fact that succinate is formed as a product of fermentation by various microorganisms, but few studies have been made, on the mechanism of its formation.

Campbell *et al.* (*2*) found, originally in *Pseudomonas aeruginosa*, that equimolar amounts of succinate and glyoxylate were formed by C_4-C_2 splitting of citrate under anaerobic condition. Later, it was established that this reaction was catalyzed by isocitritase (*3*), which was thereafter found not only in many microorganisms (*4–9*) but also in higher plants (*10–12*).

The present paper is concerned with the indentification and properties of isocitritase in *Proteus vulgaris* and with the mechanism of the anaerobic formation of succinate.

MATERIALS AND METHODS

Bacterial Cell Suspension—Proteus vulgaris grown on an ordinary pepton broth-agar slant for 18 hours at 37°C was harvested, washed by centrifugation, and suspended in deionized water or $M/50$ phosphate buffer at pH 7.2.

Cell-free Extract—Bacterial cells suspended in $M/50$ phosphate buffer (pH 7.2) were disrupted by sonic oscillation in a 9 kc Kubota vibrator for 10 minutes. Supernatant obtained by centrifugation at 12,000 r.p.m. for 15 minutes was used as the sonic extract. In some cases, the sonic extract was further fractionated by ammonium sulfate. Cell-free extract was also prepared from acetone-dried cells.

Assay of Organic Acids—Keto acids were extracted by the method of Saz and Hillary (*7*) from the reaction mixture after deproteinization with trichloroacetic acid and detected by paper chromatography and spectrophotometry. Quantitative determination of glyoxylate was performed by a method analogous to that described by Carpenter and Beevers (*12*). Succinic acid was extracted by acidic ether for 48 hours from the reaction mixture after cells or protein in cell extracts were removed by Seitz filter or deproteinization, respectively. This extract was used as samples for detection and quantitative determination. The method of detection was described in the previous paper (*1*). The quantitative determination was carried out by manometric method or by measurement of reduction of triphenyltetrazolium chloride (TTC) with succinic oxidase. Citric acid was estimated by the method of Taylor (*13*).

Estimation of Protein—Protein in the cell-free extract was estimated by the method described by Layne (*14*).

Anaerobic Condition—The experiments were always carried out in nitrogen gas or *in vacuo*.

Materials—Sodium glyoxylate was obtained from Tokyo Kasei Kogyo Co., Ltd. DL-Isocitric acid lactone and oxalacetic acid (OAA) were products of the Wako Pure Chemical Industries, Ltd. Adenosine triphosphate (ATP) was prepared by the usual method. Crude succinic oxidase was prepared from bovine heart muscle. Coenzyme A (CoA) was prepared from hog liver by the method of Lipmann (*15*) or obtained from the Sigma Chemical Company.

RESULTS

Anaerobic Formation of Succinate by Intact Cell—In the previous paper (*1*), it was shown that lactate, succinate, and an unknown volatile acid, which was neither acetic nor propionic acid, were found as the product of anaerobic metabolism of glucose by *P. vulgaris*. It is interesting that a considerable amount of succinate was formed as compared with lactate. Now, possible pathways in which succinate is produced anaerobically are illustrated in Fig. 1.

For the purpose of ascertaining by which of the pathways shown in Fig. 1 succinate formation by *P. vulgaris* was achieved, each of several intermediates was incubated with cell suspension in nitrogen gas, and after incubation, examination was made to see whether succinate could be detected or not.

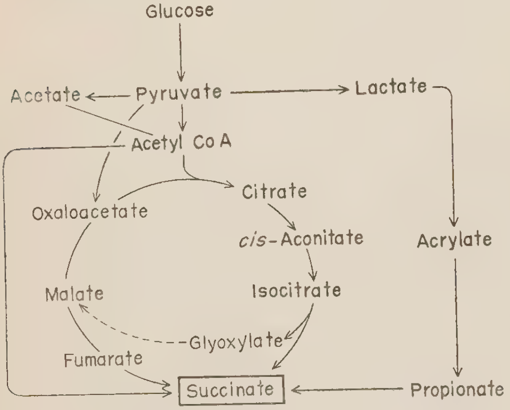

FIG. 1. Pathways of anaerobic formation of succinate.

As shown in Table I, succinate was not formed from any of the compounds except from acetate or pyruvate plus ATP.

From the above facts, the most probable pathway was considered to be the condensation of 2 moles of acetyl coenzyme A (acetyl-CoA) to form one mole of succinate. However, further examination must be made with cell-free system to see whether succinate is actually formed from citrate or not, because citrate might have been unable to permeate into bacterial cells.

TABLE I

Succinate Formation with Intact Cell Suspension

Substrate	Succinate Formation
Glucose	++++
Pyruvate	–
Acetate	–
Pyruvate+ATP (*M*/1000)	++
Acetate+ATP (*M*/5000)	++++
Citrate	–
Fumarate	–
Propionate	–
Propionate+ATP (*M*/5000)	–

Conditions: Substrate, *M*/30; phosphate buffer (pH 7.2), *M*/30; suitable amount of bacterial cell suspension; incubated at 30°C for 17 hours in nitrogen gas.

Succinate Formation by Cell-free Extract—To overcome such a factor as permeability, cell-free extract was used in later experiments. As seen in Table II, in the experiment with cell-free extract, succinate was produced from glucose or pyruvate plus ATP, as in the case of intact-cell suspension, and also from citrate. Ammonium sulfate fraction (30 to 80 per

TABLE II

Succinate Formation with Cell-free Extract

Enzyme \ Substrate	Succinate Formation			
	Glucose	Acetate+ATP (*M*/5000)	Pyruvate+ATP (*M*/5000)	Citrate
Sonic extract	++	+	+++	++
Acetone-dried powder	+		++	
Ammonium sulfate fraction				
100%	++			
0 to 30%	–			
30 to 80%	++		+++	
80 to 100%	–			
Dialyzed				
30 to 80%		++	+++	+++

Conditions: Substrate and phosphate buffer (pH 7.2), *M*/100; incubated at 30°C for 4 hours in nitrogen gas.

TABLE III

Presence of Isocitritase

Expt. No.	Enzyme	Addition	−ΔCitrate (mμmoles / mg. protein / hour)	+ΔGlyoxylate (" ")	+ΔSuccinate (" ")
1	Sonic extract		430		19.5
2	Sonic extract		423	44.4	17.1
3	Sonic extract	−KCN	408	31.8	16.8
		+KCN	433	—	41.7
4	Sonic extract	$-NaN_3$	598.3	84.8	46.2
		$+NaN_3$	652.9	84.8	50.9
5	$(NH_4)_2SO_4$ fraction (30 to 70%)		215.2	31.0	22.1
6	" "		120.0	28.8	24.0
			221.4	55.3	58.7

Conditions: Citrate, $M/100$; phosphate buffer (pH 7.2), $M/50$; suitable amount of enzyme; incubated at 30°C for 4 hours in nitrogen gas (Expt. 1 to 5) or *in vacuo* (Expt. 6).

cent saturated) of sonicates has an ability to produce succinate, but other fractions had no such ability. In these experiments, there is a possibility for production of succinate by the action of isocitritase other than the condensation reaction.

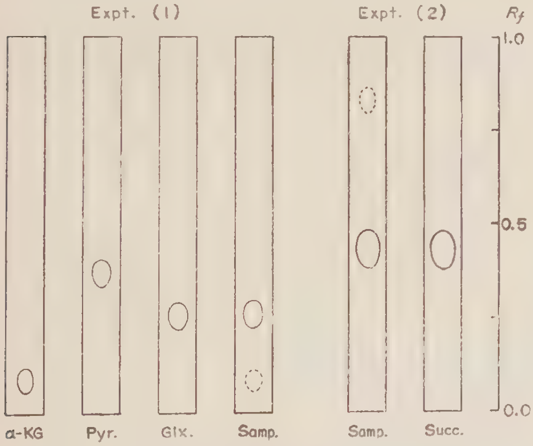

FIG. 2. Identification of reaction productsby paper chromatogram

Expt. (1)—Keto acids; solvent system 3%NH_4OH : butanol (4 : 5, *v/v*)

Expt. (2)—Non-volatile organic acids; solvent system ethanol : NH_4OH : water (80 : 5 : 15, *v/v*).

Presence of Isocitritase—If succinate was formed by the action of isocitritase, glyoxylate should be found as the product of the reaction together with succinate in the reaction mixture with citrate as the starting material. On this assumption, citrate was incubated with the sonic extract or ammonium sulfate fraction (30 to 70 per cent

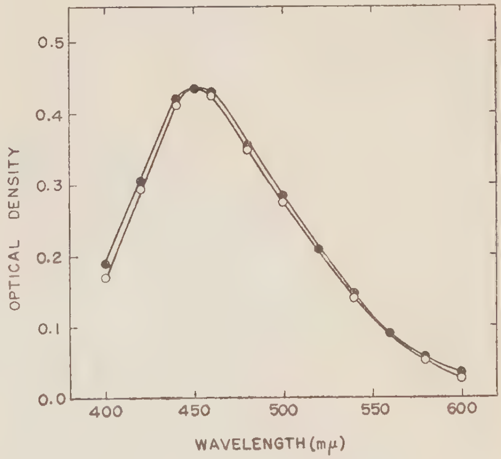

FIG. 3. Ultraviolet absorption spectra of 2,4-dinitrophenydrazones of glyoxylic acid (-○-) and reaction product (-●-) in 2 *N* sodium hydroxide

saturated) under anaerobic condition as described in the footnote to Table III. As was expected, glyoxylate was detected by paper chromatography and spectrophotometry (Figs. 2 and 3).

Though the presence of isocitritase in *P. vulgaris* was undoubted by the fact described above, examination was made to decide whether this enzyme actually exists and the amount of citrate decreased and of succinate and glyoxylate produced were estimated stoichiometrically after incubation. The results are shown in Table III.

As seen in Table III, in Expt. (1) to (5), amount of glyoxylate was found to be always more than that of succinate. This seems to be due to the consumption of a part of succinate by the presence of a small amount of oxygen contaminated in nitrogen gas. In Expt. (6), carried out *in vacuo*, equimolar glyoxylate and succinate were obtained as was expected.

Succinate Formation from Glucose by the Action of Isocitritase—The above experiments revealed that isocitritase is actually present in this bacterium and that succinate is formed from citrate by the action of isocitritase in anaerobic condition.

Then, further experiment was made in order to clarify the pathway of succinate formation from glucose, acetate, or pyruvate. If by the action of this enzyme succinate is formed from these substances, citrate must be produced in the course of the reaction besides succinate and glyoxylate. Therefore, after incubation of glucose, acetate, or pyruvate with the cell-free extract, the amounts of citrate, succinate, and glyoxylate were determined. The results are shown in Table IV. In all cases except for pyruvate, estimations were made for citrate, succinate, and glyoxylate. In the case of pyruvate, glyoxylate was detected only by paper chromatography. From the incubation of glucose, glyoxylate was not clearly detected by paper chromatography, but its formation was indubitably proved by the absorption spectrum of 2,4-dinitrophenylhydrazone of the reaction product.

When acetate was used as the starting material, formation of the reaction products was stimulated by the addition of reaction components such as CoA, ATP, and oxalacetate.

On the basis of the above results, it is assumed that the formation of succinate as a consequence of glucose metabolism under

Table IV

Existence of Citrate Condensing Enzyme

Substrate	Glucose	Pyruvate (+ATP)	Acetate (+ATP)	Acetate (+ATP CoA)		Acetate (ATP +CoA OAA)	
Enzyme / Product	Sonic extract	$(NH_4)_2SO_4$ fraction (30–70%)	$(NH_4)_2SO_4$ fraction (30–70%)				
	mμmoles per mg. protein	〃		〃	〃		
Citrate	38.5	11.8	23.9	58.8	61.9	119.1	130.1
Succinate	46.1	32.2	38.4		91.6	179.7	106.1
Glyoxylate			41.7	46.6	62.1	133.3	118.3
Reaction time (hrs.)	4	4	3	3	2	2	2

Reaction system: Substrate, *M*/100; cofactor (ATP, *M*/5000; CoA, *M*/3000; OAA, *M*/1000); phosphate buffer (pH 7.2), *M*/50; suitable amount of enzymes. In the case of citrate determination, fluoroacetate (*M*/500) was added.

Incubation: at 30C°, *in vacuo*.

anaerobic conditions was effected by the action of isocitritase.

Properties of Isocitritase—The enzyme used in the present work was prepared as follows: Sonic extract of bacterial suspension was fractionated by ammonium sulfate; the precipitate was dissolved in deionized water and dialyzed against deionized water over night at 5°C. The isocitritase activity was found to be in the fraction of 35 to 65 per cent saturation. Then the fraction was frozen at −13°C for one week. By means of this treatment aconitase involved in this preparation was almost inactivated.

Table V

Properties of Isocitritase

Enzyme: Ammonium sulfate fraction (35–65% sat.). Aconitase was inactivated by freezing at −13°C for one week.

pH Optimum: pH 7.2

Cofactor Requirement:

Reaction system	Glyoxylate (μg./mg. protein/hr.)
Complete	4.25
Minus Mg^{2+}	2.57
Minus GSH	3.35
Minus Mg^{2+}, GSH	0.67

Complete system: DL-Isocitrate, 5×10^{-3} *M*; suitable amount of enzyme; veronal buffer, 2×10^{-2} *M*; GSH (glutathione), 5×10^{-4} *M*; $MgCl_2$, 1×10^{-3} *M*.

Effect of Inhibitor:

Inhibitor	Inhibition (%)
p-CMB (1×10^{-5} *M*)	61
EDTA (1×10^{-3} *M*)	58

A. Effect of pH on the Reaction—Isocitritase activity was measured at several pH levels between 5.5 and 8.4; the maximum activity was found to be at pH 7.2.

B. Cofactor Requirement and Effect of Inhibitors—Sulfhydryl group and bivalent cation requirements for isocitritase activity have been reported by several investigators.

As shown in Table V, the activity was stimulated by glutathione and Mg^{2+}; cysteine and Mn^{2+} were also equally effective as activators. On the other hand, *p*-chloromercuribenzoate and ethylenediamine tetraacetate inhibited isocitritase activity.

DISCUSSION

In the previous paper (*1*), the authors presented a supposition that *P. vulgaris* produced succinate as a result of condensation of 2 molecules of acetyl-CoA in the process of anaerobic metabolism of glucose. The results obtained in the experiments with intact cell suspension suggested that this pathway was the most reasonable one for the formation of succinate, because propionate and fumarate (or malate), likewise citrate, were not detected in the reaction mixture as the intermediate and they were not the precursor of succinate. On the other hand, isocitritase was found in plants, fungi, and some bacteria and was partially purified, but it has not yet been reported in *P. vulgaris*. Now, the experiments with cell-free extract proved the presence of isocitritase in *P. vulgaris*. Moreover, in the experiment in which acetate, pyruvate, or glucose was incubated with cell-free extract and cofactor(s), succinate and glyoxylate were detected; it is important that citrate was recognized as an intermediate. The present results provide an evidence on the action of the key enzyme, the citrate condensation enzyme, which makes a distinction between the glyoxylate cycle and the acetyl-CoA condensation reaction. Yet, the possibility that succinate was produced by the condensation reaction together with (or besides) isocitritase can not be denied.

Though the question is not yet solved, it is difficult to consider that succinate will be formed by this condensation reaction, because it seems to be almost impossible to combine the methyl group of an acetly-CoA with the group of another acetyl-CoA. Moreover, as reported by Elsden and Peel (*16*), the velocity of a condensation reaction is very much slower than that of the succinate-splitting reaction which is the reverse reaction of the former. It may, therefore, be postulated that in *P. vulgaris*, the pathway *via* isocitritase is the main route for succinate formation from glucose at least under the anaerobic condition.

In the stoichiometric data in Table III, the amount of glyoxylate and succinate formed was always small compared to the amount of citrate consumed. Though the experimental proof for this point is lacking, it is concluded that a part of citrate consumed would be accumulated as an intermediate (*cis*-aconitate or isocitrate) from the fact that the equilibrium constant of isocitritase is favorable to the aldole condensation of succinate and glyoxy late in castor beans (*17*).

On the properties of isocitritase in *P. vulgaris*, almost similar results were obtained to those reported by several workers in other organisms except for the pH optimum (*8*, *9*, *12*). The optimum pH for *Pseudomonas aeruginosa* is at about 8.5 (*8*), while that of castor bean (*12*) is at pH 7.5.

Further studies are in progress to clarify the relationship between isocitritase and isocitric dehydrogenase activity, the biological significance of isocitritase, the possibility of a condensation reaction, etc.

SUMMARY

In the present series of work, investigations were made on the mechanism of succinate formation from glucose in *Proteus vulgaris* under anaerobic condition.

The intermediates of several pathways through which the production of succinate is considered possible were incubated with intact cells or cell-free extracts. Among citrate, fumarate, propionate, acetate, and pyruvate, succinate was formed from acetate or pyruvate plus adenosine triphosphate in the cases of both intact cell and cell-free extract, and also from citrate in the latter case.

Further, in these experiments, evidence was obtained for the presence of isocitritase in the cell-free extract of *P. vulgaris*, supporting the idea that the isocitritase played a major role in succinate formation under the present experimental conditions.

Using the ammonium sulfate fraction prepared from sonic extract of *P. vulgaris*, properties of isocitritase was examined briefly.

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Comparative Biochemical Studies on Cytochromes and Related Substances of Invertebrates

I. Cytochrome Components and Electron Transfer in Fresh-water Mussels

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Since the discovery of cytochromes by MacMunn (*1*) and by Keilin (*2*), many spectroscopic observations have been made on invertebrate cytochromes. Relatively little is known, however, of their functions and intracellular localization as compared with those of mammalian cytochromes. In a previous paper (*3*) it was reported that cytochrome *c* is the dominant cytochrome component in the heart muscle of marine bivalves, whereas in the other tissues such as gill and adductor muscle cytochrome *b* is considerably enriched in preference to cytochromes *a* and *c*. Similar facts have recently been observed with fresh-water mussels in which a *b*-type cytochrome component is abundantly present and the cytochrome patterns of different tissues differ sharply with type of tissue.

In this paper, spectroscopic and spectrophotometric observations on the cytochrome components of various cells and cellular particulates of fresh-water mussels are reported, and in addition an attempt is made to elucidate the role of the *b*-type cytochrome ("*b*") in the terminal electron-transport system.

MATERIALS AND METHODS

The fresh-water mussels used were *Cristaria plicata* and *Hyriopsis schlegelii*. They were approximately 12 to 16 cm. in shell-length, and obtained through the courtesy of a fishery association on the west coast of Lake Biwa. DPNH was purchased from the Sigma Chemical Company. Antimycin A was kindly supplied from the Kyowa Fermentation Industries Ltd., Tokyo. Cytochrome *c* was prepared from beef heart muscle by the method of Keilin and Hartree (*4*). Carbon monoxide was prepared by decomposing sodium formate with concentrated sulfuric acid.

Cell particulates were prepared from the mussel gills as follows: Excised gills were homogenized in a glass homogenizer with 5 volumes of ice-cold 0.08 M sucrose. After the homogenate was passed through a thin cloth, the filtrate was centrifuged for 10 minutes at $600\times g$; the sediment was again homogenized with 0.08 M sucrose and centrifuged in the same manner. The combined supernatants were centrifuged at $10,000\times g$ for 20 minutes to give the mitochondrial fraction. The slightly turbid supernatant was decanted; the sediment washed once by centrifugation with 0.08 M sucrose. The supernatants collected were centrifuged at $50,000\times g$ for 60 minutes in a Hitachi 40 P preparative ultracentrifuge. Microsomes were obtained as firmly packed pellets.

Direct spectroscopic observations were made with a low-dispersion hand-spectroscope on the suitable thickness of materials reduced with DPNH or sodium dithionite. The liquid-air technique of Keilin and Hartree (*5*) was also employed to intensify and sharpen the absorption bands. Difference spectra were obtained with a Cary model 14 recording spectrophotometer. Cytochrome contents were estimated from these difference spectra by applying micromolar extinction coefficients recommended by Chance (*6*). Reflectance spectra were measured with a recording Hardy type spectrophotometer (Shimadzu RC-I) at National Pearl Research Laboratory, Kashikojima. Materials suspended in 0.05 M phosphate buffer, pH 7.0, were placed in a cell of 5 mm. optical depth and of about 5 ml. capacity, which was attached to the window, 28 mm. in diameter, of integrating sphere. A white plate of magnesia soot was used as the reference.

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Abbreviation: DPNH, reduced diphosphopyridine nucleotide.

Protein was determined by the biuret (*7*) and copper-Folin (*8*) methods. Manometric measurements were made at 25°C by the conventional Warburg technique, with vessels of about 10 ml. capacity.

DPNH-cytochrome *c* reductase activity was measured by following the change in optical density at 550 mμ in a Hitachi EPU-II spectrophotometer at 25°C. The assay system contained $8\times10^{-5}M$ DPNH, $1.9\times10^{-5}M$ cytochrome *c*, $10^{-3}M$ cyanide and 0.05 *M* phosphate buffer of pH 7.0. Succinate-cytochrome *c* reductase activity was estimated in a similar manner, except that $2\times10^{-2}M$ sodium succinate was used instead of DPNH. DPNH oxidase activity was measured at 340 mμ. The experimental cuvette contained $8\times10^{-5}M$ DPNH, $1.9\times10^{-5}M$ cytochrome *c* and 0.05 *M* phosphate buffer of pH 7.0. Cytochrome oxidase activity was followed at 550 mμ. The assay system contained $1.9\times10^{-5}M$ reduced cytochrome *c* and 0.05 *M* phosphate buffer of pH 7.0. Reduced cytochrome *c* was prepared by reduction with a pinch of dithionite and the excess dithionite oxidized by shaking.

RESULTS

Direct Spectroscopic Observations—The absorption spectra observed in various tissues of *H. schlegelii* are diagramatically represented in Fig. 1. Almost identical results were also obtained in corresponding tissues of *C. plicata.* It will be seen that cytochromes are abundantly present in heart muscle, sperm and unfertilized eggs. The sperm is characterized by a typical cytochrome spectrum consisting of three α-bands due to cytochromes, *a*, *b* and *c* (Fig. 1, f). In the heart muscle, a week band corresponding to cytochrome c_1 was observed at about 554 mμ in addition to the bands of *a*, *b* and *c* at liquid-air temperature (Fig. 1, e). At room temperature, gill, mantle, adductor muscle showed only a week band at about 560 mμ, but a feeble band of cytochrome *a* and a weaker band of cytochrome *c* appeared at liquid-air temperature (Fig. 1, a ane b). The mitochondrial preparation obtained from the gill showed two intense bands at about 603 mμ and 560 mμ (Fig. 1, c). On the contrary, only one strong band was observed at 560 mμ in gill microsomes (Fig. 1, d). The band due to cytochrome *c* could not be detected both in mitochondria and microsomes even at liquid-air temperature. It is likely that cytochrome *c* has been lost from the mitochondria during preparation, since a minute amount of cytochrome *c* can be detected in the intact tissue at liquid-air temperature (Fig. 1, b). In unfertilized eggs, a strong band at 560 mμ and a week band of cytochrome *a* was observed, but cytochrome *c* was not detectable even at liquid-air temperature (Fig. 1, g). The α-band of cytochrome *b* in the sperm and the heart muscel appeared at about 562 mμ. In this respect, the cytochrome *b* of sperm and heart muscle seems to differ from a *b*-type cytochrome of unfertilized eggs and others.

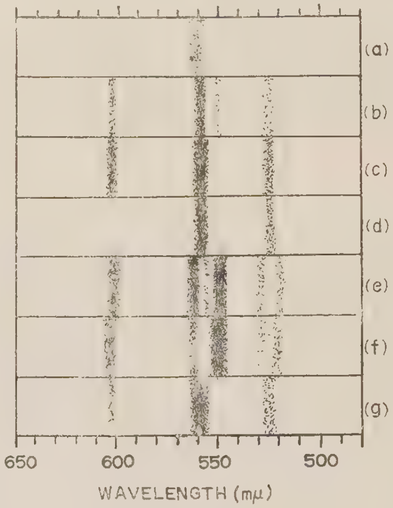


Fig. 1. Diagram showing the cytochrome spectra of *H. schleglii.* (a) Gill pieces with $Na_2S_2O_4$ at room temperature. (b) The same at −186°C. (c) Gill mitochondria with $Na_2S_2O_4$ or with DPNH at −186°C. (d) Gill microsomes with $Na_2S_2O_4$ at −186°C. (e) Heart muscle with $Na_2S_2O_4$ at −186°C. (f) Sperm suspension with $Na_2S_2O_4$ at room temperature. (g) Unfertilized eggs with $Na_2S_2O_4$ at room temperature.

Spectrophotometric Observations on Intact Cells and Particulate Preparations—The observations described above were further confirmed by spectrophotometric experiments. Typical examples of the reflectance and difference spectra are illustrated in Figs. 2 to 5. Under the reduced state, the sperm suspension showed absorption maxima at 418, 520. 550 and 603 mμ (Fig. 2). In the aerobic suspension, the only distinct peak appeared at about 412 mμ. The absorption maxima at 418 and 412 mμ, under reduced and oxidized conditions respectively, may be mainly attributed to the γ-band of cytochrome *c*.

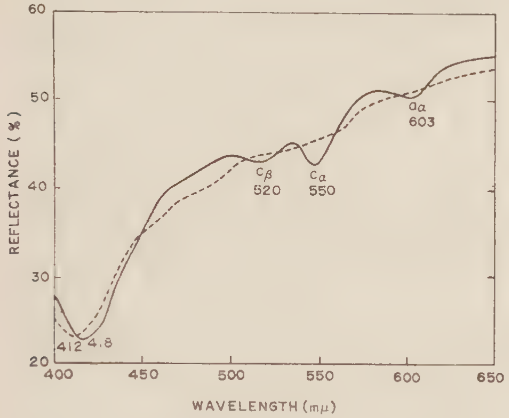


Fig. 2. Reflectance spectra of sperm suspensions of *H. schlegelii.* Dotted trace, aerobic suspension. Solid trace, suspension reduced with $Na_2S_2O_4$. Sperm cells 35 mg. dry weight per ml. The materials were suspended in 0.05 *M* phosphate buffer, pH 7.0.

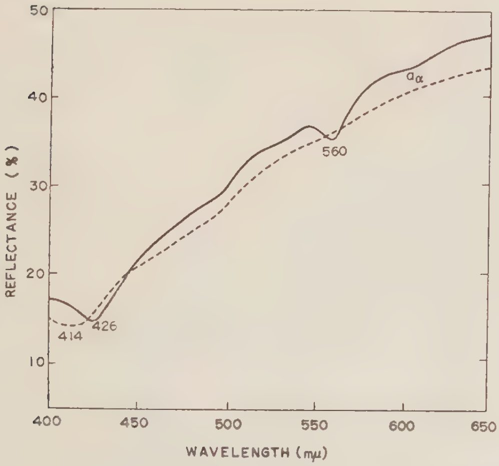


Fig. 3. Reflectance spectra of unfertilized eggs of *H. schlegelii.* Dotted trace, aerobic suspension. Solid trace, egg suspension reduced with $Na_2S_2O_4$. Eggs 73 mg. dry weight per ml.

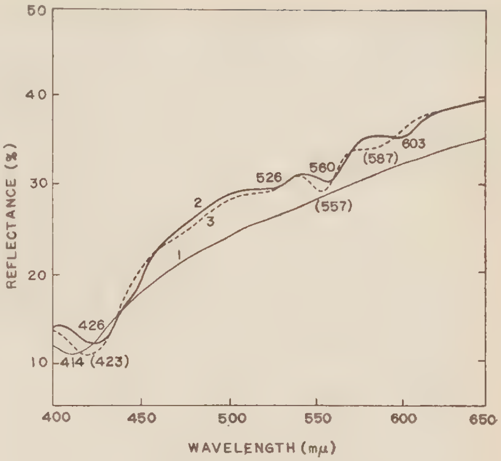


Fig. 4. Reflectance spectra of gill mitochondria of *H. schlegelii.* 1, aerobic suspension of mitochondria. 2, reduced with $Na_2S_2O_4$. 3, added $Na_2S_2O_4$+pyridine. Mitochondria 27 mg. dry weight per ml.

A cytochrome component having an α-band at 560 mμ is abundantly present in unfertilized eggs (Fig. 3) and cell particulates of gills (Fig. 4). This cytochrome is in spectroscopic properties similar to those of the so-called "cytochrome b_1" which occurs in certain micro-organisms. The unfertilized eggs contained, in addition to the *b*-type cytochrome, a trace of cytochrome *a*. Gill mitochondria showed a peak at 414–416 mμ in the oxidized state, and four peaks at 426, 526, 560 and 603 mμ when reduced with dithionite (Fig. 4). The absorption peaks of pyridine haemochromogen of mitochondrial cytochromes lie at 423, 525, 557 and 587 mμ. The peaks at 557 and 587 mμ can be attributed to the α-band of pyridine haemochromogens of the *b*-type cytochrome and cytochrome *a*, respectively.

The "*b*" component in gill microsomes was readily reduced by DPNH under anaerobic conditions (Figs. 5, 2), but not reduced by succinate. However, the "b" component in mitochondria was reduced by succinate as readily as by DPNH. When the mitochondria suspension which had once been anaerobically reduced with DPNH or succinate in the presence of cyanide was shaken with air, though the peaks of cytochrome *a* remained unoxidized, those of the *b*-type cytochrome were rapidly diminished (Fig. 6). From the data of reflectance and difference spectra, the absorption peaks of the *b*-type cytochrome in the reduced form is estimated to lie at 426–

428 mμ (Soret, 412 mμ in the oxidized form), 526 mμ (β) and 560 mμ (α).

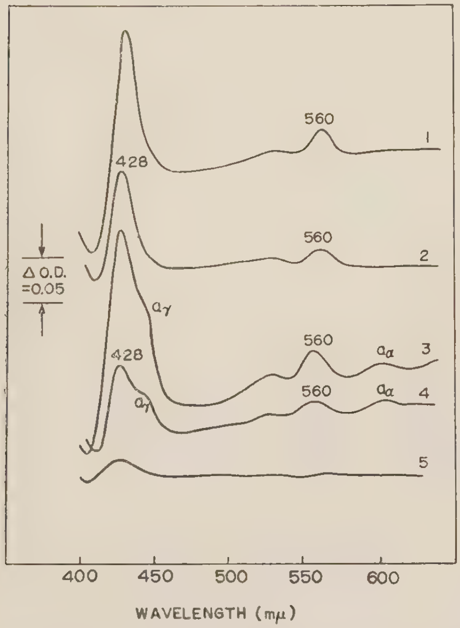


Fig. 5. Spectra representing the difference in absorbancy between the reduced and oxidized cytochrome components of cell particulates of *H. schlegelii*. 1, Difference between the $Na_2S_2O_4$-reduced gill microsomes and the aerobic microsomes. 2, between the DPNH-added anaerobic and the aerobic microsomes. 3, between the $Na_2S_2O_4$-reduced and the aerobic gill mitochondria. 4, between the DPNH-added anaerobic and the aerobic gill mitochondria. 5, between the DPNH-added aerobic and the aerobic gill mitochondria. Light path of cuvettes, 1 cm. The materials were suspended in 0.05 *M* phosphate buffer of pH 7.0. Concentration : gill microsomes 9.6 mg. protein per ml., gill mitochondria 6.3 mg. per ml.

Concentrations of Cytochrome Components—The concentrations of cytochromes in various cells and cell particulates are summarized in Table I. The heart muscle and sperm contained considerable amounts of cytochrome *c*. Assuming that the mean value of protein contents in fresh matters is 16 per cent, based on the estimation of water contents, the content of cytochrome $c+c_1$ in whole heart muscle was calculated to be approximately 3.6×10^{-5} moles per liter. This value is comparable to that obtained with mammalian heart muscle (*9*). In the gill, the *b*-type cytochrome seems to be distributed between mitochondria and microsomes in similar concentrations.

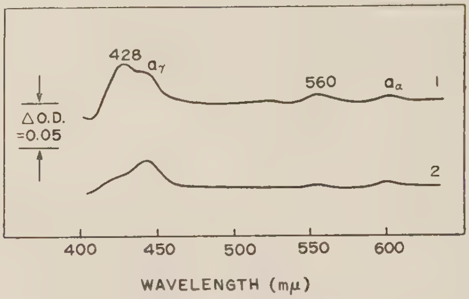


Fig. 6. Oxidation of the *b*-type cytochrome of gill mitochondria in the presence of cyanide. 1, Difference spectrum between the anaerobic mitochondria treated with DPNH+cyanide and the aerobic mitochondria. 2, the same, after shaking with air. The mitochondria were suspended in 0.05 *M* phosphate buffer of pH 7.0. Mitochondria 3.7 mg. protein per ml.

Table I

Cytochrome Components of Whole Cells and Cell Particulates

Material	Contents of cytochromes in mμmoles per mg. protein			
	a	"*b*"[1]	*c*	$c+c_1$
H. schlegelii				
sperm	0.07[2]	0.04[2]	0.15[2]	—
unfertilized eggs	trace	0.11	—	—
hert muscle	0.06	0.13	—	0.20
gill mitochondria	0.11	0.23	—	—
gill microsomes	—	0.21	—	—
C. plicata				
gill mitochondria	0.11	0.15	—	—
gill microsomes	—	0.12	—	—

1) include normal cytochrome *b*.
2) mμmoles per mg. dry weight.

Intracellular Distribution of Respiratory Enzymes—Experiments were carried out using the cell fractions of *H. schlegelii* (Table II). Although DPNH-cytochrome *c* reductase was widely distributed among the three cytoplasmic fractions, the activities of cytochrome oxidase and succinate-cytochrome *c* reductase were found only in the mitochondrial fraction.

The mitochondrial succinate-cytochrome *c* reductase system was completely inhibited by 2 μg. per ml. of antimycin A, but DPNH-cytochrome *c* reductase of all cytoplasmic fractions was unaffected by antimycin A at the concentrations of 2 to 10 μg. per ml. The oxidation of DPNH by the mitochondrial fraction, as measured at 340 mμ, did not proceed in the absence of added cytochrome *c*, and its activity was inhibited by cyanide and anti-

TABLE II

Enzymatic Activities in Cell Fractions

Cell fraction	Cytochrome oxidase1)	DPNH oxidase2)	DPNH-cyt. *c* reductase	Succinate-cyt. c reductase3)
Mitochondrial	275	685	485	42
Microsomal	9	0	358	0
Soluble	0	0	408	0

1) mμmoles cytochrome *c* oxidized per mg. N per minute.
2) mμmoles DPNH oxidized per mg. N per minute.
3) mμmoles cytochrome *c* reduced per mg. N per minute.

TABLE III

Effect of Inhibitors on the Enzymatic Activities of Gill Mitochondria

Inhibitor	Inhibition (per cent)			
	Cytochrome oxidase	DPNH oxidase	DPNH-cyt. *c* reductase	Succinate-cyt. *c* reductase
Cyanide $10^{-3}M$	94	58	—	—
Antimycin *A*				
2 μg. per ml.	—	—	0	99
10 μg. per ml.	—	20	0	100

TABLE IV

Effect of Cyanide on the Endogenous Respiration of the Whole Cells

Material	Control O_2 μl./100 mg. fresh weight/hr.	Cyanide $5\times10^{-4}M$ Inhibition (per cent)	No. of experiments
H. schlegelii gill	16–20	51	5
C. plicata, gill	26–28	59	4
H. schlegelii, sperm	63–90¹⁾	79	3

1) O_2 μl./10^{10} cells per hr.

The oxygen consumption was measured manometrically at 25°C. Each flask contained 200 mg. of thin gill pieces or about 8×10^9 cells of sperm in 1.5 ml. of mussel Ringer solution (*11*) containing 0.002 *M* phosphate buffer, pH 7.0. In the cyanide experiments, 0.2 ml. of KCN-KOH mixture (5.5 *M* KCN in 0.5*M* KOH) (*12*) was placed in the center well.

mycin A. Mitochondrial cytochrome oxidase was strongly inhibited by cyanide. The oxidase activity measured manometrically was also inhibited by carbon monoxide in the dark and this inhibition was almost completely eliminated by the illumination with a 500-watt projector lamp (Fig. 7).

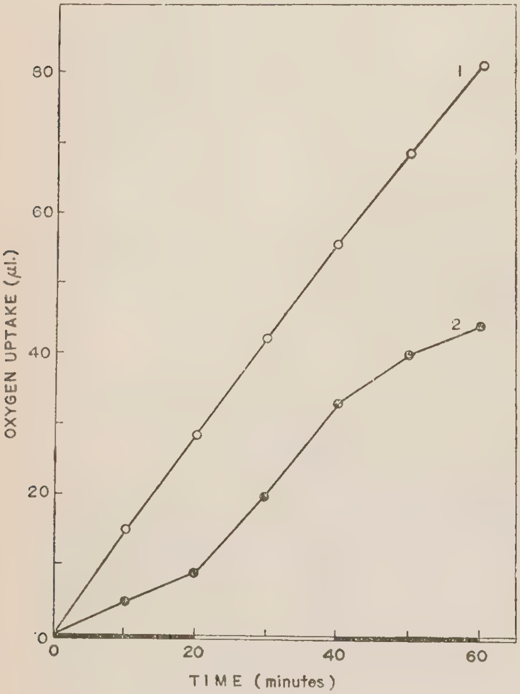


FIG. 7. Effect of carbon monoxide on cytochrome oxidase from *H. schlegelii*. Each flask contained the followings in 2.0 ml. of 0.05 M phosphate buffer, pH 7.0: 8.8 mg. protein of gill mitochondria, 20 μmoles ascorbate, 30 mμmoles cytochrome *c*, 1, in N_2-O_2 (95 : 5). 2, in CO-O_2 (95 : 5). The black and white blocks under the base line show the periods of dark and light.

Respiration of Whole Tissue—The rate of endogenous respiration of intact gill was about 1.3–1.8 as expressed in terms of Q_{O_2}. Although the respiratory activity of gill is the highest among the tissues tested as reported previously (*10*), the observed rate is considerably lower than those of higher animals. As shown in Table IV, the respiration of intact gill was relatively insensitive to cyanide. Cyanide, at a concentration of $5\times10^{-4}M$, depressed the oxygen consumption of the gill to about 40–50 per cent of the control value, and this inhibition was reversed by the addition of $10^{-3}M$ of methylene blue. On the contrary, the same concentration of cyanide depressed the oxygen consumption of the mussel sperm to about 20 per cent of the control. In a gas mixture of 95% CO and 5% O_2, the respiration of the gill was inhibited by about 55 per cent in the dark and this inhibition was almost completely eliminated by light (Fig. 8).

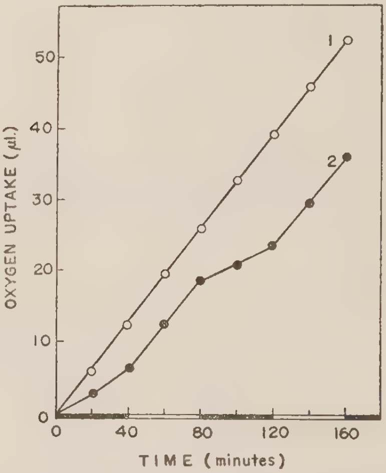


FIG. 8. Effect of carbon monoxide on the endogeneous respiration of gill pieces. 1, in N_2-O_2 95 : 5). 2, CO-O_2 (95 : 5). Each flask contained 100 mg. gill pieces of *H. schlegelii* in 1.5 ml. of mussel Ringer solution (*11*) containing 0.002 M phosphate buffer of pH 7.0. The white blocks under the base line show the periods of illumination.

DISCUSSION

Although the fresh-water mussels examined here are equiped with no oxygen carrier such as haemoglobin or haemocyanin in their blood, they contain relatively large amounts of cytochromes in their tissues as compared with marine invertebrates which have so far been examined by the present author. Recently, a cytochrome-like haemoprotein called enterochrome 566 has been found in the gut fluids of these fresh-water bivalves, and some of its properties has been established with purified preparations (*13*), though the physiological role of the haemoprotein is as yet unknown. As is well known, these bivalves usually live in the muddy bottom of ponds and lakes, where dissolved oxygen is very rare owing to the oxidation of sedimented organic matters by bacterial actions. Generally speaking, it seems to have been fairly established the biosynthesis of haemoproteins by an organism is more active in the surrounding media of low oxygen pressure than in those of normal oxygen supplies (*14–16*). It seems, therefore, reasonable to assume that the high cytochrome contents of the mussels is closely related to the poor supply of oxygen in their natural habitats.

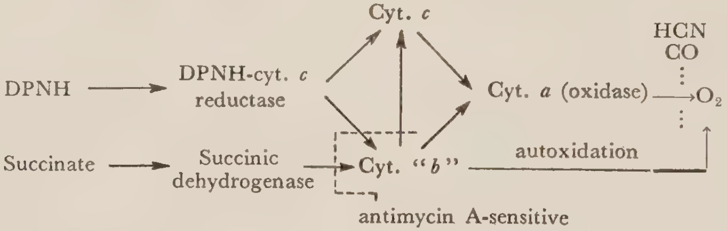
It was reported in a previous paper (*3*) that the concentrations of various cytochrome components in the tissues of marine bivalves are considerably different according to the types of tissues. Similar variations in the cytochrome patterns have also been more clearly demonstrable in fresh-water mussels. Based on the cytochrome patterns, the mussel cells may be classified into three categories, *i.e.* heart muscle, gill, and unfertilized-egg types. Heart muscle and sperm belong to the first type and are characterized by high concentrations of cytochromes *a*, *b* and *c*; cytochrome *c* being predominant over cytochromes *a* and *b*. The second type exemplified by gill includes adductor muscle, mantle, *etc.*, in which the *b*-type cytochrome is considerably richer than cytochrome *a* and the content of cytochrome *c* is negligible. Among the tissues examined, only unfertilized eggs belong to the third type which is particular in the high contents of the "*b*" component and complete absence of cytochrome *c*. It is of interest that the cytochrome composition in sperm is in a striking contrast with that in unfertilized eggs, although the biological significance of the phenomenon is to be explored.

The *b*-type cytochrome is localized in similar concentrations in both the mitochondrial and microsomal fractions of gill. In addition to the "*b*" component, the mitochondrial fraction contains a small amount of cytochrome *a*. In the microsomal fraction, on the other hand, the "*b*" component is the only detectable cytochrome. In view of its intracellular distribution, the "*b*" component should be classified in the so-called microsomal cytochromes. The absorption characteristics of the *b*-type cytochrome component are in many respects similar to those of microbial cytochrome b_1 and a cytochrome found in a particulate preparation of *Ascaris* muscle (*17*).

Investigations of certain respiratory enzymes of gill cytoplasmic fractions indicated that the mitochondrial particles are the chief site of cell respiration, as in the case of mammalian cells. However, the cytochrome components of the gill mitochondria markedly differ from those of mammalian liver or heart muscle mitochondria. Thus, in contrast to mammalian tissue, the intact tissue contains only a negligible amount of cytochrome *c* and this component is observable only at liquid-air temperature. No trace of cytochrome *c* can be detected in the mitochondria prepared therefrom. As already mentioned, it is probable that cytochrome *c* has been released from the mitochondria during the fractionation of the cells. Recently, it has been described that certain cell particulates of marine molluscs contain cytochrome components very similar to those of mammalian mitochondria (*18*, *19*). In the present investigation, however, the similarity to mammals in cytochrome components was confirmed with the mussels only in the heart muscle and sperm. Detailed studies on the cytochrome components and electron transfer in the mussel sperm will be reported elsewhere.

The respiration of intact gill is relatively insensitive to cyanide as compared with that of the mussel sperm in which cytochrome *c* is predominantly present. The "*b*" component of gill mitochondria, being rapidly reduced by DPNH or succinate under the anaerobic conditions, is readily oxidized by oxygen in the presence of cyanide. This fact suggests that the *b*-type cytochrome is autoxidable and that it may play a part in the cyanide-insensitive respiration like cytochrome b_5 in the midgut of *Cecropia* silk-worm (*20*) and a cytochrome *b* component in the *Ascaris* muscle (*17*). The pathway of electron transfer from succinate to cytochrome *c* in the gill mitochondria is apparently associated with the antimycin A-sensitive factor, although it is not clear wheather the factor is located between the *b*-type cytochrome and cytochrome *c* or the factor constitutes a part of the *b*-type cytochrome. The results of spectrophotometric experiments with gill mitochondria further suggest that a cyanide-sensitive pathway of electron transport is operating *via* cytochrome *a* from the "*b*" to oxygen apart from the cyanide-insensitive autoxidation of the "*b*" cytochrome.

Summarizing all the findings obtained, it may be possible to suggest the following scheme as the pathways of electron transfer in the gill and other tissues having the "*b*" component in relatively high concentrations.



The physiological significance of she *b*-type cytochrome present in the gill microsomes in high concentrations is not yet clear and would be an interesting problem like that of cytochrome b_5 in mammalian liver microsomes (*21*).

SUMMARY

The cytochrome composition in the tissues of fresh-water mussels markedly differs according to the types of tissues. Cytochrome *c* is richly contained in heart muscle and sperm, and a *b*-type cytochrome is abundantly present in unfertilized eggs, gill, adductor muscle, *etc.* The absorption maxima of the reduced *b*-type cytochrome in whole cells and cell particulates lie at 560 mμ (α), 526 mμ (β) and 426–428 mμ (Soret, 412 mμ in she oxidized form). The "*b*" component is autoxidable.

The mitochondrial fraction of the gill abundantly contains the "*b*" component and *a*, whereas the microsomal fraction contains the "*b*" as the only detectable cytochrome. The "*b*" component in the mitochondria is reduced by DPNH as readily as succinate under anaerobic condition. The "*b*" in the microsomes is readily reduced by DPNH, but not reduced by succinate. The distribution of cytochrome oxidase and succinate-cytochrome *c* reductase are restricted in the mitochondria, while DPNH-cytochrome *c* redmctase is widely distributed among cytoplasmic fractions. The DPNH-cytochrome *c* reductase is not inhibited by antimycin A, but the succinate-cytochrome *c* reductase is completely inhibited by antimycin A.

A scheme is proposed to account for the possible pathways of electron transfer in the molluscan tissues containing the *b*-type cytochrome in high concentrations.

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Synthesis and Properties of Serotonin O-Sulfate

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Serotonin (5-Hydroxytryptamine) is widely distributed in organisms and known to have various physiological functions. In mammals it is regarded as one of chemical neurotransmitters.

According to Chadwick and Wilkinson (*1*), serotonin can be converted to serotonin O-sulfate by rat liver homogenates. Serotonin O-sulfate was found in urine when a large quantity of serotonin was injected to rats and rabbits (*2*).

These findings led us to the working hypothesis that intracellular serotonin level might be regulated, at least partly, by the mutual change of serotonin and serotonin O-sulfate. From the point of view, serotonin O-sulfate was synthesized and some of its properties were investigated.

MATERIALS AND METHODS

Estimation of Serotonin—The colorimetric method with p-dimethylaminobenzaldehyde for tryptophan estimation was applied (*3*). The extinction was measured at 560 mμ.

Estimation of Sulfate—The benzidine sulfate method according to Dodgson and Spencer (*4*).

Detection of Sulfate Ester on Paper Chromatography—Sulfate ester on paper chromatogram was located with rhodizonate reagent according to Schneider and Lewbart (*5*).

Measurement of Contraction of Rat Ileum by Drugs—Isolated rat ileum (small intestine) was suspended in a tube with Ringer solution and its contraction by drugs was recorded on kymographion by the use of isotonic lever.

Preparation of Arylsulfatases—Ox liver, rabbit liver and rabbit brain: 1 g. of the acetone powder of each organ was incubated with 100 ml. of acetate buffer (0.5 *M*, pH 6.5) for an hour at 37°C. The suspension was centrifuged at 22,000×*g* and the supernatant was used as enzyme preparation (*6*). *Charonia lampas* (*Tritonalia sauliae*) liver: The crude extract of the acetone powder was treated with trypaflavin and CM-cellulose according to Takahashi (*7*).

Preparateon of Substrates—Serotonin O-sulfate was synthesized as described below. Potassium nitrocatechol sulfate (potassium 2-hydroxy-5-nitrophenyl sulfate) was prepared according to Roy (*8*) and potassium tyramine O-sulfate according to Dodgson (*9*).

Determination of Enzyme Activities—The hydrolysis of nitrocatechol sulfate was measured by colorimetric estimation of the liberated nitrocatechol (*8*). The hydrolysis of serotonin O-sulfate and tyramine O-sulfate was measured by estimation of liberated sulfate by the benzidine sulfate method (*4*).

RESULTS

Synthesis of Serotonin O-Sulfate—Serotonin creatinine sulfate (2 g.) was added in small portions at 15°C into the mixture of sulfuric acid (10 ml.) and chlorosulfonic acid (1.3 ml.) under stirring. The reaction mixture was placed for an hour at 15°C and then hydrogen chloride was removed under reduced pressure in the cold. The reaction mixture was poured into 100 ml. of crushed ice and water, and neutralized with barium carbonate. After filtration the solution was passed through a column (10 cm.×1 cm.) of Amberlite IR 120 ion-exchange resin (H^+form) which adsorbs serotonin unchanged. The effluent was lyophilized and the residue was dissolved in a small amount of water. The solution was neutralized with KOH solution and kept in the cold. 28 mg. (3 per cent of calculated yield) of sulfate ester of serotonin, colorless needles, separated out. Recrystallized from water.

When sulfuric acid only was used without chlorosulfonic acid, the yield was less than 1 per cent of calculated. Three kinds of acidic by-products not yet identified were found when the reaction temperature rose to 37°C or the reaction mixture was kept overnight at room temperature.

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Analysis—The synthesized substance contained serotonin and sulfate at the molar ratio of 1 : 1.

1. Analysis with the Solution—The solution with the synthesized substance was hydrolyzed with 2 *N* HCl at 100°C for 1.5 hours. With the solution serotonin and sulfate were estimated. 1 ml. of the solution contained 3.35 μmoles of serotonin and 3.36 μmoles of sulfate.

TABLE I

R_f Values of Serotonin O-Sulfate and Serotonin

Solvent system[1]	R_f value	
	Serotonin O-sulfate	Serotonin
A	0.31	0.46
B	0.57	0.34
C	0.38	0.68
D	0.65	0.69

1) A : Butanol-acetic acid-water (4 : 1 : 5, by vol.)
B : 20% KCl (w/v)
C : Propan-2-ol-aqueous 6 *N* ammonia (20 : 3, v/v)
D : Butanol-pyridine-water (1 : 1 : 1, by vol.)

Chromatography was performed by the ascending technique on Toyo No. 51A paper at 20°C.

The spray used for detecting compounds on paper was Ehrlich's reagent (*10*): 1 g. of *p*-dimethylaminobenzaldehyde/90 ml. of acetone plus 10 ml. of conc. HCl.

2. Analysis of the Crystal—The crystal was dried *in vacuo* at 60°C for two hours, weighed and dissolved in water. The method of analysis was above-mentioned.

	Observed	Calculated as $C_{10}H_{12}O_4N_2S$	Calculated as $C_{10}H_{12}O_4N_2S,2H_2O$
Serotonin :	60.3%	68.8%	60.7%
Sulfur :	10.4%	12.5%	11.0%

The difference between the observed value and the calculated might be explained by assuming the existance of two molecules of water of crystallization.

Paper Chromatography—Serotonin sulfate can be separated from serotonin by paper chromatography. R_f values of both compounds are shown in Table I. Color of spots with Ehrlich's reagent was pink for ester and blue for serotonin itself. The spot of sulfate ester marked with rhodizonate reagent agreed with the spot given with Ehrlich's reagent.

Since this sulfate ester of serotonin gave the color reaction with ninhydrin but not with 1-nitroso-2-naphthol and nitrous acid which is characteistic of 5-hydroxyindoles (*11*), it is phenolic hydroxyl group that was sulfated.

From the data of analysis and paper-chromatography the synthesized substance is regarded as serotonin O-sulfate with the structure of Fig. 1. Infrared and ultraviolet spectra mentioned below also supported this structural formula.

FIG. 1. Serotonin O-sulfate.

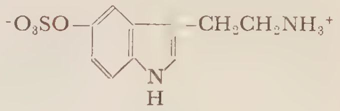


TABLE II

Contraction of Rat Ileum by Serotonin O-Sulfate and Serotonin

Concentration of drug in bath (*μg./ml.*)	Shortening on the graph (*mm.*)	
	Serotonin O-sulfate	Serotonin
10	52	53
1	10	60
0.1	not detectable	46
0.01		17
0.001		not detectable

Infrared Spectrum of Serotonin O-Sulfate—Two strong bands at 1050 and 1250 cm.$^{-1}$ and a broad absorption at 3000–3500 cm.$^{-1}$ indicate the extance of S–O and -NH_3^+ groups respectively (Fig. 2).

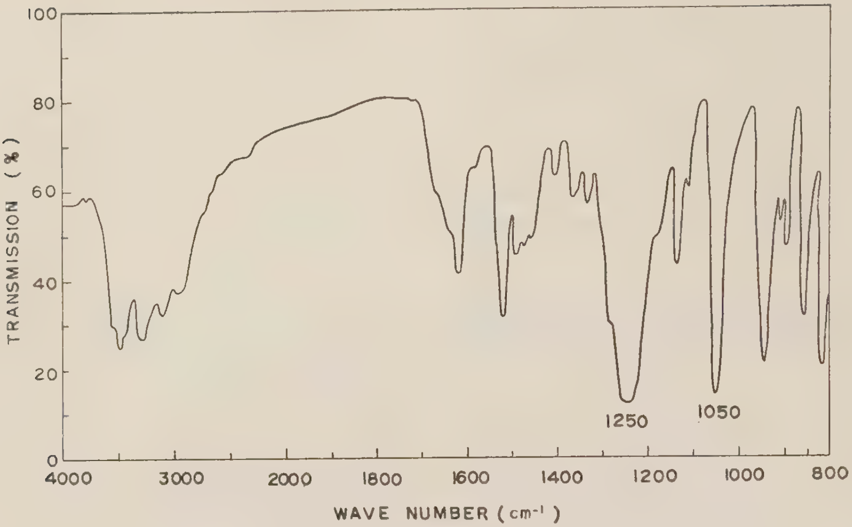


FIG. 2. Infrared spectrum of serotonin O-sulfate.
Hitachi-EPI-2 IR spectrophotometer with NaCl prism. Sample was pressed in KBr.

TABLE III

Hydrolysis of Serotonin O-Sulfate and Other Arylsulfates by Arylsulfatases

Enzyme	Protein (mg.)	Substrate[1]	(µmoles)	Substrate hydrolyzed 1 hour (µmoles)	1 hour %	24 hours (µmoles)	24 hours %
Charonia lampas liver	0.5	NCS	10	6.2	62		
	0.4	TOS	5	0.57[2]	11		
	2.0	SOS	8	0.60	7.7	2.4	31
Ox liver	1.7	NCS	40	0.52	1.3		
	1.7	TOS	20			0.52	2.6
	1·7	SOS	6			0	0
Rabbit liver	1.8	NCS	10	0.48	4.8		
	1.8	TOS	20			0.25	1.3
Rabbit brain	0.9	NCS	10	0.37	3.7		
	0.9	TOS	20			0.19	1.0
	0.9	SOS	6			0	0

1) NCS, nitrocatechol sulfate; TOS, tyramine O-sulfate; SOS, serotonin O-sulfate
2) Tris-HCl buffer (0.04 *M*, pH 7.0)

Reaction mixture: enzyme preparation, substrate and acetate buffer (0.1–0.15 *M*. pH 6.0) Total volume was 0.5–1.0 ml.

Incubation at 37°C.

Ultraviolet Spectra of Serotonin O-Sulfate and Serotonin—The spectrum of serotonin O-sulfate is almost independent of pH, while the spectrum of serotonin varies appreciably with pH. It may be due to the phenolic group in the latter (Fig. 3).

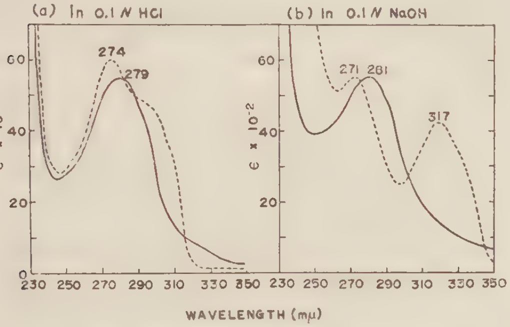


Fig. 3. Ultraviolet spectra of serotonin O-sulfate and serotonin.

Serotonin O-sulfate—, serotonin···

(a) in 0.1 *N* HCl (b) in 0.1 *N* NaOH

Beckmann-DK2 spectrophotometer.

Pharmacological Properties of Serotonin O-Sulfate—Table II shows the shortening of rat ileum at varied concentration of serotonin O-sulfate and serotonin.

Since the shortening of ileum by 1 μg./ml. of O-sulfate is less than that by 0.01 μg./ml. of serotonin, it is concluded that the contraction activity of the former is, if any, less than one hundredth of the latter. The minute activity of O-sulfate might be due to serotonin as an impurity.

Hydrolysis of Serotonin O-Sulfate by Arylsulfatases—Table III shows experimental conditions and hydrolysis of nitrocatechol sulfate, tyramine O-sulfate and serotonin O-Sulfate by various enzyme preparations.

Serotonin O-sulfate was hydrolyzed by arylsulfatase of *Charonia lampas*, though the activity for it was less than one fortieth for nitrocatechol sulfate. Mammalian arylsulfatases in the experimental conditions did not hydrolyze serotonin O-sulfate even in 24 hours' incubation.

DISCUSSION

Serotonin O-sulfate was synthesized and its properties were investigated. They can be well explained by the structural formula in Fig. 1.

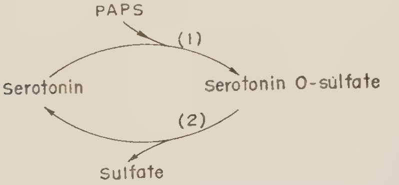
Contrary to serotonin, O-sulfate was found to be almost inactive in the contraction of rat ileum. This is not surprising because the artivity largely depends on the phenolic hydroxyl group in serotonin. For example, the activity ratio of guinea-pig intestine by serotonin analogues are: serotonin, 100; 5-methoxytryptamine, 33; tryptamine, 0.17 (*12*).

After completion of the experiments described in this report, there appeared a paper by Chadwick and Wilkinson on biochemical studies of serotonin O-sulfate (*13*). It was reported that they could not succeed in the synthesis of serotonin O-sulfate. We also failed in the synthesis many times because of the unsuitable operation, when brown pigments and many by-products were formed. In spite of various attempts we could not get better yield than described here.

It should be pointed out that R_f values of our synthetic serotonin O-sulfate do not agree so well with R_f values of the natural substance tentatively identified by them as serotonin O-sulfate. The reason remains to be elucidated.

Concerning enzymatic hydrolysis of serotonin O-sulfate, they found that it was hydrolyzed by acetone powder of rat liver, but not by its soluble extract. In agreement with them our soluble extract of acetone powder of mammalian organs did not hydrolyze serotonin O-sulfate. However, mammalian arylsulfatase activities of the above extract for nitrocatechol sulfate and tyramine O-sulfate being quite low, the possibility of the enzymatic hydrolysis of serotonin O-sulfate with much higher enzyme concentration or with particulate preparations (arylsulfatase C (*6*)) cannot be excluded. It is noteworthy that it was hydrolyzed by a soluble sulfatase in the case of *Charonia lampas*.

They also reported that when rats were pretreated with 1-isopropyl-2-isonicotinylhydrazide phosphate (an inhibitor of monoamine oxidase), the large doses of serotonin were mostly converted to serotonin O-sulfate. This may be a process of detoxication for the excess of serotonin. However, because serotonin O-sulfate can be hydrolyzed by sulfatases as shown by them and us, it is not excluded that it participates in the *in vivo* regulation of serotonin level by the following cyclic process. Metabolic process in the conversion of serotonin to O-sulfate (process 1) has been investigated by Goldberg (*14*) and Chadwick (*13*). The process 2 is catalysed by an arylsulfatase. The regulation of hormone concentration through sulfate ester has been also suggested in the case of thyroid hormone by Roche *et al.* (*15*).



PAPS: 3'-phosphoadenosine-5'-phosphosulfate

SUMMARY

1. Serotonin O-sulfate was synthesized and its infrared spectrum, ultraviolet spectrum and paper chromatographic properties were investigated.

2. On rat small intestine the contraction (or stimulating) activity of serotonin O-sulfate was, if any, less than one hundredth of serotonin.

3. Serotonin O-sulfate was hydrolyzed by sulfatase of *Charonia lampas* (*Tritonalia sauliae*), but not measurably by the extracts of mammalian organs.

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The Journal of Biochemistry, Vol. 49, No. 5, 1961

Intracellular Distribution of D-Amino Acid Oxidase in Rat Liver

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It is well known that D-amino acid oxidase is contained in the kidney and the liver (*1*, *2*), however, no well-defined physiological meaning for this enzyme has been known in animal tissues. As a mean to elucidate the physiological action of this enzyme, the intracellular distribution of the enzyme was examined.

Although Paigen reported the existence of this enzyme in several types of mitochondria in rat liver (*3*), we intended to examine in detail the distribution of this enzyme with each subcellular fraction. The existence of the enzyme was ascertained by extracting it from a sub- cellular fraction.

MATERIALS AND METHODS

Albino rats weighing about 100 g. were used as experimental animals. For the fractionation of the mitochondria and supernatant of the liver, the method of Schneider and Hogeboom (*4*) was used. For the isolation of nuclear fraction, rat liver weighing about 2 g. was perfused first with 0.145 M NaCl and then with 0.25 M sucrose containing 0.0018 M $CaCl_2$. The liver was then minced with scissors and homogenized in 0.25 M sucrose containing 0.0018 M $CaCl_2$. In order to remove connective tissue and large clumps of liver cells, the homogenate was filtered through two layers of gauze. A 20 ml. aliquot of the filtered homogenate was layered carefully over 40 ml. of 0.34 M sucrose containing 0.00018 M $CaCl_2$ in a 60 ml. test tube as described by Hogeboom (*5*), and the mixture was centrifuged for 10 minutes at 600×g for 10 minutes at 0°C. The supernatant was removed, and the sediment was homogenized for 15 seconds in 10 ml. of 0.25 M sucrose containing 0.00018 M $CaCl_2$. Twenty ml. of 0.34 M sucrose containing 0.00018 M $CaCl_2$ were then introduced slowly beneath the suspension of nuclei, and the mixture was centrifuged again at 600×g for 10 minutes at 0°C. The procedure of homogenization of the pellet, layering, and centrifugation was repeated twice more.

D-Amino acid oxidase activity of cell fraction was measured by a conventional Warburg manometer using the following reaction mixture: 5×10^{-2} M D-alanine, 5×10^{-4} M KCN, 5×10^{-6} M FAD* prepared by our method (*6*) (in their final concentration) and suspension of each subcellular fraction in 2.0 ml. of 2.5×10^{-2} M pyrophosphate buffer (pH 8.3). Center well contained 0.2 ml. of 20% KOH. After the temperature equilibrium had been reached, D-alanine was mixed from the side bulb, and the readings were taken.

To test the contamination of the mitochondria in other fractions, the activity of succinic dehydrogenase was measured by a conventional Warburg manometer using the reaction mixture containing $1\times10^{-1}$$M$ succinate, $2\times10^{-3}$$M$ $MgCl_2$, $2\times10^{-2}$$M$ KCl, $2\times10^{-3}$$M$ ATP, $3\times10^{-2}$$M$ glucose (in their final concentration), 300 μg./ml. hexokinase and suspension of each subcellular fraction in 2.0 ml. of $4\times10^{-3}$$M$ phosphate buffer (pH 7.4). After the temperature equilibrium had been reached, glucose and hexokinase were mixed from the side bulb, and the readings were taken.

RESULTS AND DISCUSSION

The oxygen uptake of the homogenate and nuclear, mitochondrial and supernatant fractions in the above-mentioned reaction mixture was shown in Table I.

To confirm the above activities to D-amino acid oxidase, purification of the enzyme from each subcellular fraction of rat liver was carried out according to the method of Negelein and Brömel (*8*), and it was followed up to step 3, where all the catalase activity and

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* Abbreviations: FAD, flavin adenine dinucleotide; ATP, adenosine triphosphate.

TABLE I

The Oxygen Uptake of Subcellular Fractions

Substrate	D-Alanine				Succinate			
Subcellular fraction	Homogenate	Nuclear fraction	Mitochondrial fraction	Supernatant	Homogenate	Nuclear fraction	Mitochondrial fraction	Supernatant
Q_{O_2} (N)[1]	49.5 0.36 :	10.7 0.08 :	137.8 1 :	43.6 0.32	221.5 0.12 :	70.8 0.04 :	1800.0 1 :	107.6 0.06

[1] Q_{O_2} (N): μl. O_2 Uptake/hour/mg. nitrogen of the suspension of each subcellular fraction. Nitrogen was measured by an indophenol method (*7*).

almost all the FAD were separated from the oxidase protein. In these enzyme solutions, the oxygen uptake was only measured by the addition of both D-alanine and FAD, and no oxygen uptake was observed by the addition of only D-alanine or FAD. The ratio of the activities of the enzyme solutions purified from the nuclear, mitochondrial and supernatant fractions was nearly the same as that of the activities of these fractions themselves. From these results, the oxygen uptake measured with each subcellular fraction may be attributed to D-amino acid oxidase activity.

The distribution of activity of D-amino acid oxidase in nuclear, mitochondrial, and supernatant fractions, showed 0.08 : 1 : 0.32 expressed by Q_{O_2} (N) as shown in Table I. Then, the distribution of the activity of succinic dehydrogenase in each fraction showed 0.04 : 1 : 0.06, respectively. Assuming that the activity of succinic dehydrogenase runs parallel with the amount of the mitochondria contaminated, the ratio of the distribution of activity of D-amino acid oxidase in each subcellular fraction was corrected to be 0.04 : 1 : 0.26, respectively.

These numbers indicated that the enzyme existed chiefly in the mitochondrial fraction and small amount in the supernatant, but negligible in the nuclear fraction.

The activity in the supernatant was rather high. However, whether this enzymatic activity of the supernatant can be attributed to its original existence or emigration from the mitochondria during the preparation of cell fraction may be remained for further investigation.

These results indicate that D-amino acid oxidase in rat liver is concentrated in its mitochondria.

The present experiment indicates that this enzyme is rather easily extracted from the mitochondria. This result is in accordance with the fact that the effect of added FAD on the aged mitochondria was easily demonstrated in the case of D-amino acid oxidase activity (*9*).

SUMMARY

The oxygen uptake by nuclear, mitochondrial, and supernatant fractions of rat liver in D-amino acid oxidase reaction mixture was measured. Then the apo-enzyme of D-amino acid oxidase was purified from each fraction, and the activity was tested.

The ratio of the activities of D-amino acid oxidase purified from the nuclear, mitochondrial, and supernatant fractions was nearly the same as that of the activities of these fractions themselves.

The ratio of the enzyme activity in these subcellular fractions was corrected by the contamination of the mitochondria, calculated by the ratio of succinic dehydrogenase activity.

The results showed that the larger amount of D-amino acid oxidase exists in the mitochondria, and the small amount in the supernatant.

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Biosynthesis of Isovalthine in the Cat

Mizuhara *et al.* (*1*), (*2*) isolated a new sulfur-containing amino acid from the urine of hypercholesterolemic patients. The chemical structure of tho amino acid was established as S-(isopropyl-carboxymethyl) cysteine by synthesis and the amino acid was named isovalthine (*3*), (*4*).

Isovalthine was also found in the urine of cats, and a study on its biosynthesis was carried out by the administration of leucine to cats.

Two per cent solution of L-leucine was administered to three cats by a stomach tube for three days. The total amount of leucine used was 1.86 g. The urine of cats was collected in a bottle containing toluene and concentrated hydrochloric acid for five days each, before and after leucine administrations, and a total of 850 ml. of urine sample was obtained from each cat. The apholyte fraction in the urine was collected by using ion exchangers as described in the previous paper (*2*). In the present experiment, hydrolysis of urine was carried out before using ion exchangers. Solvent systems used for paper chromatography of the urinary ampholyte fraction were butanol-acetic acid-water (4 : 1 : 4 v/v) and phenol-water (4 : 1 v/v, 0.2% NH_3 in the cell).

Fig. 1 shows the pattern of urinary amino acid of the cat before leucine administration and Fig. 2 shows the pattern after leucine intake. A new large spot appeared at R_f 0.53 and 0.45, as seen in Fig. 2. The new spot was positive to sulfur tests on paper and was identified as isovalthine by the comparison of R_f values with authentic samples such as isovalthine and its analogues.

Since the concentration of urinary isovalthine is always lower than that of other amino acids, isovalthine is seldom found on paper chromatogram containing all the urinary amino acids, as shown in Fig. 1, but it is seen in almost every pattern of acidic amino acid fraction prepared by treating amino acid mixture with weakly acidic resin (*e.g.* Amberlite CG-45) as described previously (*2*).

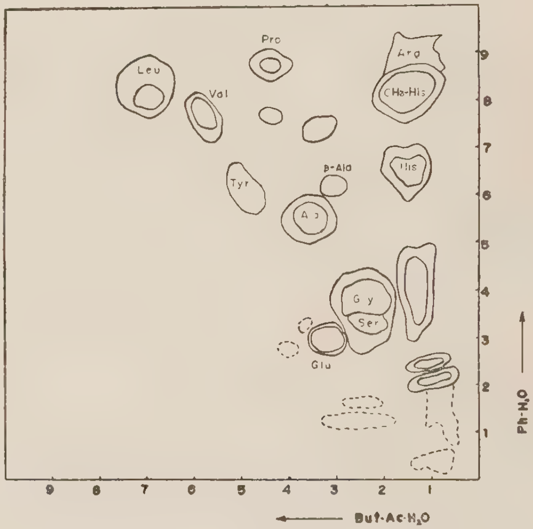

Fig. 1. Amino acids in urine of cat (normal).

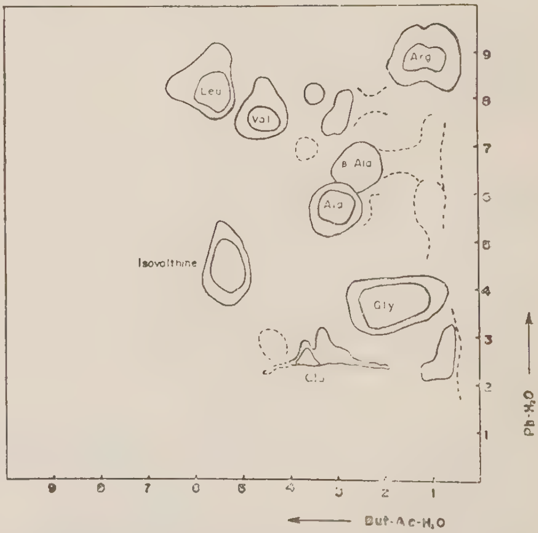

Fig. 2. Amino acids in urine of cat (leucine administered)

After leucine intake, however, a large spot of isovalthine is found even in the chromatogram containing all the urinary amino acids (Fig. 2).

Therefore it may be considered that isovalthine is synthesized from leucine in the cat.

An effort is now being made to obtain a more definite proof of the above implication by using radioactive leucine.

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Rapid Excretion of Choleglobin in Urine after Intravenous Administration of Hemoglobin to Rabbits

Hemoglobin has been shown to break down to choleglobin, then to verdohemoglobin when it was incubated in the presence of ascorbic acid and molecular oxygen (*1*, *2*). Verdohemoglobin, when treated with HCl, quantitatively yields biliverdine (*3*). Thus the reaction of hemoglobin breakdown in the system mentioned has been considered to be the best model of hemoglobin catabolism *in vivo*. However, it is not clear as yet whether the *in vivo* metabolism of hemoglobin would actually proceed in similar fashion as in the model system. In this paper, we wish to report our findings that, after intravenous administration of hemoglobin to rabbits, substantial amounts of choleglobin, together with hemoglobin, were excreted in urine at a high rate.

Destromatized rabbit hemoglobin in physiological saline was infused through auricular vein and thereafter urines were collected by catheterization at intervals of about 30 minutes. An aliquot of each urine sample was mixed with 3% NaOH and water at the ratio of 1 : 1 : 1. Insoluble substances were centrifuged off, and the resultant clear supernatant was added with a pinch of pulverized $Na_2S_2O_4$ and examined spectrophotometrically.

As shown in Fig. 1, A (Solid line), there was a distinct absorption maximum at 618 mμ in addition to protohemochrome spectrum in the region of 500–600 mμ. The peak at 618 mμ shifted to 630 mμ when the sample was gassed with CO (Fig. 1, A—broken line). In Fig. 1, B, spectra are given of another sample which was not treated with NaOH, but with the addition of $Na_2S_2O_4$. There can be seen a strong absorption by reduced hemoglobin and an additional absorption indicating its maximum at 630 mμ (Fig. 1, B—solid line). The 630 mμ absorption became more distinctive when the sample was passed with CO (Fig. 1, B—broken line). These spectral characteristics shown in Fig. 1 coincied exactly with those of authentic choleglobin (*1*, *2*). We were unable to distinguish spectrophotometrically the presence of verdohemoglobin in urine, but we could obtain small amounts of biliverdine from urine by applying procedures for extracting biliverdine (*1*, *2*). Durations of hemoglobin and choleglobin excretion differed between individual experiments, but usually were three to four hours. There was noticed a tendency that amounts of choleglobin excreted were less when rates of hemoglobin excretion were lower. Per

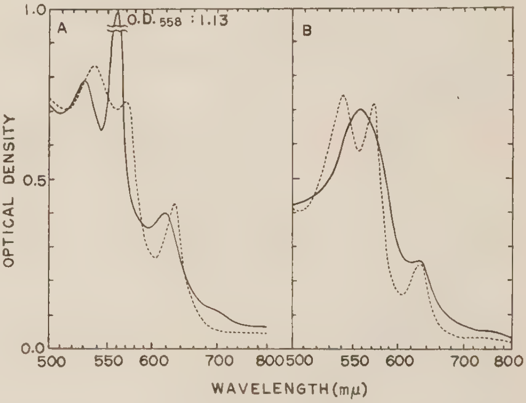

Fig. 1. Absorption spectra of urine samples from rabbits which were intravenously administered with hemoglobin.

A: Hemoglobin administered contained 19 μmoles heme. Initial 30 minutes urine sample was used. Solid line: treated with NaOH and $Na_2S_2O_4$. Broken line: CO-gas was passed through above sample.

B: Hemoglobin administered contained 47 μmoles heme. Intial 35 minutes urine sample was used. Solid line: urine sample was diluted with equal volume of water and reduced with $Na_2S_2O_4$. Broken line: CO-gas was passed through above sample.

cent contents of choleheme in total heme in urine samples were calculated according to the empirical equations presented by Kaziro *et al.* (*2*), and compared in Table I.

TABLE I

Per cent Contents of Choleheme in Total Heme in Urine Samples

(from the same series of experiments as presented in Fig. 1, B).

Time in min. after the hemoglobin administration	Per cent contents of choleheme
35	22
70	18
100	22
130	30
160	40

The examination of circulating blood of a rabbit at 35 and 70 minutes after the intravenous administration of hemoglobin revealed also the appearance of choleglobin in the blood plasma, although ratios of choleheme to protoheme were found to be far lower as compared with those in urine samples.

It is of particular interest to note that significant amounts of choleglobin were formed in the living body and excreted into urine within as short as 30 minutes after the administration of hemoglobin. The results would indicate that the hemoglobin metabolism *in vivo* may proceed in a similar way as in the model system as far as reaction products and sequences are concerned. Also the high rate of hemoglobin breakdown *in vivo* thus observed could account for Yamada's observation (*4*) that, when hemoglobin was intravenously administered to rabbits, the biliverdine excretion into fistula bile increased rapidly showing a small initial lag, and the maximum rate was reached at about two hours after the hemoglobin administration. In contrast to rapid formation of choleglobin *in vivo*, only trace amounts of choleglobin were formed during *in vitro* incubation of hemoglobin with citrated whole blood of rabbits at conditions as designed to be comparable to those in *in vivo* experiments. This would suggest that the reaction of hemoglobin breakdown *in vivo* may be participated by activities of living cells, although their roles in the reaction are not known. Further studies are in progress with special concerns to the presumed participation of living cells in the *in vivo* metabolism of hemoglobin.

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Further Studies on Soluble Lactonase

Identity of Lactonase I and Aldonolactonase with Gluconolactonase

Presence of an enzyme activity hydrolyzing the lactone of uronic acids and aldonic acids was reported by several workers in the past. Gluconolactonase which hydrolyzes gluconolactone 6-phosphate to the corresponding acid was found by Brodie and Lipmann (*1*) in bacteria, yeast, and rat liver homogenate. Presence of an enzyme activity hydrolyzing D-glucuronolactone in the rat liver homogenate was reported by Eisenberg and Field (*2*). Winkelman and Lehninger (*3*) reported the occurrence of two distinct lactonases, aldonolactonase and uronolactonase, differing each other in tissue distribution, intracellular location, and substrate specificity. In their findings these enzymes did not appear to be identical with the gluconolactonase. Yamada *et al.* (*4*, *5*) reported independently the presence of two distinct lactonases, named lactonase I and II. Lactonase I was found in the supernatant fraction and lactonase II, in the microsomal fraction of liver homogenates. These two enzymes differed in their substrate specificity and alkali lability. Yamada (*6*) further observed that lactonase I catalyzed lactonization of L-gulonic acid and D-glucuronic acid, and showed that lactonase I has an important role in ascorbic acid biosynthesis.

The present paper deals with the identification of lactonase I and aldonolactonase with gluconolactonase.

Lactonase I was prepared and purified from acetone-dried powder of bovine liver by the method of Yamada (*5*). The enzyme activity for the lactone of uronic acids and aldonic acids except for D-glucono-δ-lactone was assayed by the manometric method described in Yamada's paper (*5*), and for D-glucono-δ- and D-gulono-γ-lactone by the following colorimetric method.

The standard reaction mixture consisted of enzyme solution, 10 μmoles of substrate (D-glucono-δ- or D-gulono-γ-lactone), 1 μmole of $MgSO_4$, 50 μmoles of phosphate buffer (pH 6.0 or 7.0), and water which was added to make the total volume of 1.0 ml. The mixture was incubated at 37°C for 5 minutes and analyzed for hydroxamic acid formation. A blank test was carried out without the enzyme. To the reaction mixture, 2.0 ml. of the neutralized hydroxylamine reagent (1.5 M) was added and mixed. After 20 minutes at 37°C, 0.5 ml. each of conc. HCl, 12% trichloroacetic acid, and 60% $FeCl_3$, were added. If precipitate was formed, it was removed by centrifugation and the colored supernatant solution was read after 20 minutes on a colorimeter at 540 mμ. A standard curve was plotted at each experiment. One unit is defined as the amount of enzyme which hydrolyzes 0.1 μmole of the substrate per minute.

The ratio of the activity of D-glucono-δ-lactone to D-gulono-γ-lactone, and of D-gulono-γ-lactone to D-glucurono-γ-lactone was constant at about 5.7 and 7.9 respectively for each step of purification of the enzyme. Furthermore, each eluate from DEAE-cellulose column also gave a similar pattern in respect to the enzyme activity for D-glucono-δ-, D-gulono-γ-, and D-glucurono-γ-lactones. Therefore, a single enzyme may be responsible for these lactone-hydrolyzing activities.

The substrate specificity of purified lactonase was examined at pH 6.0 and 7.0 as shown in Table I. Almost the same result was obtained by using rat liver supernatant instead of purified lactonase.

At pH 6.0, specificity of the present enzyme was in agreement with the reported specificity of gluconolactonase (*1*) except for the activity on D-galactono-γ-lactone. When several volumes of the enzyme were used, the activity for D-gulono-γ-, and D-galactono-γ-lactone was detected at the same pH. At pH 7.0, when the volume of the enzyme solution for the assay of D-glucono-δ-lactone was used, the specificity of the present enzyme also agreed with gluconolactonase (*1*) except for the activity on D-galactono-γ-lactone, while by the use of 5 volumes of the enzyme, the pattern of specificity of the present enzyme closely resembled the reported pattern of lactonase I (*5*) and aldonolactonase (*3*), except for the activity on D-glucono-δ-lactone. This discrepancy of the activity on D-glucono-δ-lactone may be due to the difference of the assay methods used. The activity for this lactone could not be assayed correctly by the manometric method, because the lactone was rapidly hydrolyzed nonenzymatically at pH 7.0 during preincubation. It has been reported that aldonolactonase could not hydrolyze D-glucuronolactone (*3*), but it did in the present series of experiments. Lactonase I is similar to gluconolactonase in that its activity was greatly stimulated by the presence of Co^{++}, Mg^{++}, and Mn^{++}.

TABLE I

Substrate	Enzyme activity (unit)	
	pH of the reaction mixture	
	pH 6.0	pH 7.0
D-Glucono- -lactone	48 [240] [a]	96 [480]
D-Gulono-γ-lactone	0 [28]	0 [86]
D-Galactono-γ-lactone	0 [16	0 [62]

Reaction mixture contained purified enzyme, 0.02 ml.; substrate, 10 μmoles; $MgSO_4$, 1 μmoles, and phosphate buffer, 50 μmoles.
Total volume 1.0 ml.; pH 6.0 or 7.0.
Incubation 5 minutes at 37°C.

a) [ ] indicates the activity when 5 volumes of the enzyme was used.

The above findings suggest that lactonase I purified from acetone-dried powder of bovine liver, gluconolactonase from rat liver homogenate, and aldonolactonase from soluble fraction of rat liver are the same enzyme. Similarly, the liver of human and monkey (*Macaca cynomolga Linne*) showed lactone-hydrolyzing activity for aldonic acids and uronic acids, but the activity was slight.

The writers thank Dr. K. Yamada for his valuable advice throughout this investigation.

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